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**WO 03/064626 A2**

**(54) Title:** DOUBLE-STRANDED OLIGONUCLEOTIDES

**(57) Abstract:** Antisense sequences, including duplex RNAi compositions, which possess improved properties over those taught in the prior art are disclosed. The invention provides optimized antisense oligomer compositions and method for making and using the both in *in vitro* systems and therapeutically. The invention also provides methods of making and using the improved antisense oligomer compositions.

## DOUBLE-STRANDED OLIGONUCLEOTIDES

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### **Related Applications**

This application claims the priority of U.S. provisional patent application no. 60/353,203, filed on February 1, 2002, application no. 60/436,238, filed December 23, 2002, and application no. 60/438,608, filed January 7, 2003. This application also claims the priority of 10 60/353,381, filed February 1, 2002. The entire contents of the aforementioned applications are hereby expressly incorporated herein by reference.

### **Background of the Invention**

Complementary oligonucleotide sequences are promising therapeutic agents and 15 useful research tools in elucidating gene function. However, oligonucleotide molecules of the prior art are often subject to nuclease degradation when applied to biological systems. Therefore, it is often difficult to achieve efficient inhibition of gene expression (including protein synthesis) using such compositions.

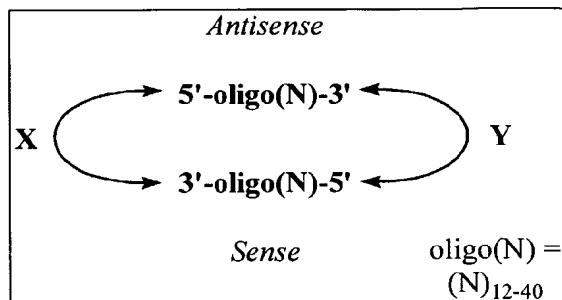
In order to maximize the usefulness, such as the potential therapeutic activity and *in* 20 *vitro* utility, of oligonucleotides that are complementary to other sequences of interest, it would be of great benefit to improve upon the prior art oligonucleotides by designing improved oligonucleotides having increased stability both against serum nucleases and cellular nucleases and nucleases found in other bodily fluids.

### **Summary of the Invention**

The instant invention is based, at least in part, on the discovery that double-stranded oligonucleotides comprising an antisense oligonucleotide and a protector oligonucleotide, are capable of inhibiting gene function. Thus, the invention improves the prior art antisense sequences, *inter alia*, by providing oligonucleotides which are resistant to degradation by 30 cellular nucleases.

Accordingly, the invention provides optimized oligonucleotide compositions and methods for making and using both in *in vitro*, and *in vivo* systems, *e.g.*, therapeutically.

In one aspect, the invention pertains to a double-stranded oligonucleotide composition having the structure:



5

*Figure 1.*

where (1) N is a nucleomonomer in complementary oligonucleotide strands of equal length and where the sequence of Ns corresponds to a target gene sequence and (2) X and Y are each independently selected from a group consisting of nothing; from about 1 to about 20 nucleotides of 5' overhang; from about 1 to about 20 nucleotides of 3' overhang; and a loop structure consisting from about 4 to about 20 nucleomonomers, where the nucleomonomers are selected from the group consisting of G and A.

An "overhang" is a relatively short single-stranded nucleotide sequence on the 5'- or 3'-hydroxyl end of a double-stranded oligonucleotide molecule (also referred to as an "extension," "protruding end," or "sticky end").

In one embodiment, the number of Ns in each strand of the duplex is between about 12 and about 50 (*i.e.*, in the figure above, oligo(N) has between about 12 and about 50 nucleomonomers). In other embodiments, the number of Ns in each strand of the duplex is between about 12 and about 40; or between about 15 and about 35; or more particularly between about 20 and about 30; or even between about 21 and about 25.

In one embodiment, X is a sequence of about 4 to about 20 nucleomonomers which form a loop, wherein the nucleomonomers are selected from the group consisting of G and A.

In one embodiment, two of the Ns are unlinked, *i.e.*, there is no phosphodiester bond between the two nucleomonomers. In one embodiment, the unlinked Ns are not in the antisense sequence.

In one embodiment, the nucleotide sequence of the loop is GAAA.

In another aspect, the invention pertains to a double-stranded oligonucleotide composition having the structure:

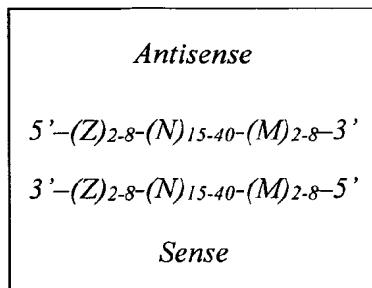


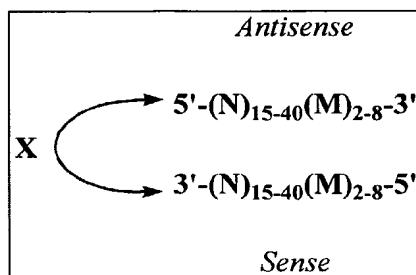
Figure 2.

5 where (1) N is a nucleomonomer in complementary oligonucleotide strands of equal length  
where the sequence of Ns corresponds to a target gene sequence; and (2) Z is a  
nucleomonomer in complementary oligonucleotide strands of between about 2 and about 8  
nucleomonomers in length and where the sequence of Zs optionally corresponds to the target  
sequence; and (3) where M is a nucleomonomer in complementary oligonucleotide strands of  
10 between about 2 and about 8 nucleomonomers in length and where the sequence of Ms  
optionally corresponds to the target sequence. Although the sequences of N nucleomonomers  
should be of the same length, the sequences of Z and M nucleomonomers may optionally be  
of the same length.

15 In one embodiment, Z and M are nucleomonomers selected from the group consisting  
of C and G.

In one embodiment, the sequence of Zs or Ms is CC, GG, CG, GC, CCC, GGG, CGG,  
GCC, GCG, CGC, CGGG, GCCC, CCCC, GGGG, GCGC, CGCG, GGGC, CCCG, CGGG,  
GCCG, GGCC, or CCGG.

20 In another aspect, the invention pertains to a double-stranded oligonucleotide  
composition having the structure:

*Figure 3.*

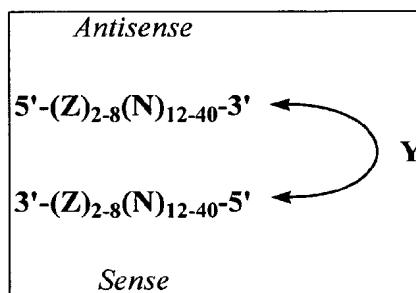
- 5 where (1) N is a nucleomonomer in complementary oligonucleotide strands of equal length and where the sequence of Ns corresponds to a target gene sequence and (2) X is selected from the group consisting of nothing; 1-20 nucleotides of 5' overhang; 1-20 nucleotides of 3' overhang.

10 In some embodiments, X is a loop structure consisting of from about 4 to about 20 nucleomonomers, where the nucleomonomers are selected from the group consisting of G and A.

15 In the structure above, M is a nucleomonomer in complementary oligonucleotide strands of between about 2 and about 8 nucleomonomers in length which optionally correspond to the target sequence. In one embodiment, M is a nucleomonomer selected from the group consisting of C and G.

In one embodiment, the sequence of M is CC, GG, CG, GC, CCC, GGG, CGG, GCC, GCG, CGC, CGGG, GCCC, CCCC, GGGG, GCGC, CGCG, GGGC, CCCG, CGGG, GCCC, GGCC, or CCGG.

20 In another aspect, the invention pertains to a double-stranded oligonucleotide composition having the structure:

*Figure 4.*

where (1) N is a nucleomonomer in complementary oligonucleotide strands of equal length and which correspond to a target gene sequence and (2) Y is selected from the group consisting of nothing; 1-20 nucleotides of 5' overhang; 1-20 nucleotides of 3' overhang; a loop consisting of a sequence of from about 4 to about 20 nucleomonomers, where the  
5 nucleomonomers are all either Gs or A's and (3) where Z is a nucleomonomer in complementary oligonucleotide strands of between about 2 and about 8 nucleomonomers in length and which comprise a sequence which can optionally correspond to the target sequence.

In one embodiment, Zs are nucleomonomers selected from the group consisting of C  
10 and G.

In one embodiment, the sequence of Zs is CC, GG, CG, GC, CCC, GGG, CGG, GCC, GCG, CGC, CGGG, GCCC, CCCC, GGGG, GCGC, CGCG, GGGC, CCCG, CGGG, GCCC, GGCC, or CCGG.

In another aspect, the invention pertains to a method of regulating gene expression in  
15 a cell, comprising forming a double-stranded oligonucleotide composition as described herein and contacting a cell with the double-stranded duplex, to thereby regulate gene expression in a cell.

In one embodiment, the invention pertains to a method of increasing the nuclease  
resistance of an antisense sequence, comprising forming a double-stranded oligonucleotide  
20 composition as described herein, such that a double-stranded duplex is formed, wherein the nuclease resistance of the antisense sequence is increased compared to a double-stranded, unmodified RNA molecule.

Methods of stabilizing oligonucleotides, particularly antisense oligonucleotides, by formation of a oligonucleotide compositions comprising at least 3 different oligonucleotides,  
25 are disclosed in co-pending application no. U.S. \_\_\_\_\_, filed on the same day as the present application, bearing attorney docket number "SRI-013," and entitled "Oligonucleotide Compositions with Enhanced Efficiency." This application and all of its teachings is hereby expressly incorporated herein by reference in its entirety.

### 30 Brief Description of the Drawings

Figure 1 shows that the length of double-stranded oligonucleotides and the presence or absence of overhangs has no effect on function.

Figure 1B shows the effect of structural changes on the efficacy of siRNAs targeting β-3-Integrin.

Figure 2 shows that there is no correlation was observed between the length of the double-stranded oligonucleotide and the level of PKR induction for the given sequences.

Figure 2B shows effect of  $\beta$ -3-integrin targeted 21-mer and 27-mers on PKR expression in HMVEC Cells.

5 Figure 3 shows the effect of 5' or 3' modification on activity of double-stranded RNA duplexes.

Figure 4 shows the effect of the size of the modifying group on activity of the double-stranded RNA duplex.

10 Figure 5 shows the results of 2'-O-Me modifications on the activity of double-stranded RNA duplexes.

Figure 6 shows the inhibition of p53 by 32- and 37-mer blunt-end siRNAs.

### Detailed Description of the Invention

The instant invention advances the prior art by providing double-stranded 15 oligonucleotide compositions for use, both *in vitro* and *in vivo*, e.g., therapeutically, and by providing methods of making and using the double-stranded antisense oligomer compositions.

#### Double-stranded Oligonucleotide Compositions

20 Double-stranded oligonucleotides of the invention are capable of inhibiting the synthesis of a target protein, which is encoded by a target gene. The target gene can be endogenous or exogenous (e.g., introduced into a cell by a virus or using recombinant DNA technology) to a cell. As used herein, the term "target gene" includes polynucleotides comprising a region that encodes a polypeptide or polynucleotide region that regulates 25 replication, transcription, translation, or other process important in expression of the target protein; or a polynucleotide comprising a region that encodes the target polypeptide and a region that regulates expression of the target polypeptide; or non-coding regions such as the 5' or 3' UTR or introns. Accordingly, the term "target gene" as used herein may refer to, for example, an mRNA molecule produced by transcription a gene of interest. Furthermore, the 30 term "correspond," as in "an oligomer corresponds to a target gene sequence," means that the two sequences are complementary or homologous or bear such other biologically rational relationship to each other (e.g., based on the sequence of nucleomonomers and their base-pairing properties).

The “target gene” to which an RNA molecule of the invention is directed may be associated with a pathological condition. For example, the gene may be a pathogen-associated gene, *e.g.*, a viral gene, a tumor-associated gene, or an autoimmune disease-associated gene. The target gene may also be a heterologous gene expressed in a recombinant cell or a genetically altered organism. By determining or modulating (*e.g.*, inhibiting) the function of such a gene, valuable information and therapeutic benefits in medicine, veterinary medicine, and biology may be obtained.

The term “oligonucleotide” includes two or more nucleomonomers covalently coupled to each other by linkages (*e.g.*, phosphodiesters) or substitute linkages. In one embodiment, it may be desirable to use a single-stranded nucleic acid molecule which forms a duplex structure (*e.g.*, as described in more detail below). For example, in one embodiment, the oligonucleotide can include a nick in either the sense or the antisense sequence.

The term “antisense” refers to a nucleotide sequence that is inverted relative to its normal orientation for transcription and so expresses an RNA transcript that is complementary to a target gene mRNA molecule expressed within the host cell (*e.g.*, it can hybridize to the target gene mRNA molecule through Watson-Crick base pairing). An antisense strand may be constructed in a number of different ways, provided that it is capable of interfering with the expression of a target gene. For example, the antisense strand can be constructed by inverting the coding region (or a portion thereof) of the target gene relative to its normal orientation for transcription to allow the transcription of its complement, (*e.g.*, RNAs encoded by the antisense and sense gene may be complementary). Furthermore, the antisense oligonucleotide strand need not have the same intron or exon pattern as the target gene, and noncoding segments of the target gene may be equally effective in achieving antisense suppression of target gene expression as coding segments.

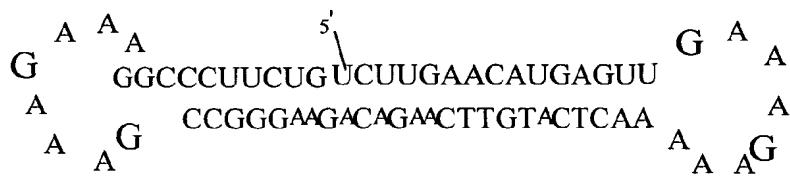
Accordingly, one aspect of the invention is a method of inhibiting the activity of a target gene by introducing an RNAi agent into a cell, such that the dsRNA component of the RNAi agent is targeted to the gene. In one embodiment, an RNA oligonucleotide molecule may contain at least one nucleomonomer that is a modified nucleotide analogue. The nucleotide analogues may be located at positions where the target-specific activity, *e.g.*, the RNAi mediating activity is not substantially effected, *e.g.*, in a region at the 5'-end or the 3'-end of the double-stranded molecule, where the overhangs may be stabilized by incorporating modified nucleotide analogues.

In another aspect, double-stranded RNA molecules known in the art can be used in the methods of the present invention. Double-stranded RNA molecules known in the art may also be modified according to the teachings herein in conjunction with such methods, *e.g.*, by using modified nucleomonomers. For example, *see* U.S. 6,506,559; U.S. 2002/0,173,478 A1; 5 U.S. 2002/0,086,356 A1; Shuey, *et al.*, "RNAi: gene-silencing in therapeutic intervention." Drug Discov. Today 2002 Oct 15;7(20):1040-6; Aoki, *et al.*, "Clin. Exp. Pharmacol. Physiol. 2003 Jan;30(1-2):96-102; Cioca, *et al.*, "RNA interference is a functional pathway with therapeutic potential in human myeloid leukemia cell lines. Cancer Gene Ther. 2003 Feb;10(2):125-33.

10 Further examples of double-stranded RNA molecules include those disclosed in the following references: Kawasaki, *et al.*, "Short hairpin type of dsRNAs that are controlled by tRNA(Val) promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells." Nucleic Acids Res. 2003 Jan 15;31(2):700-7; Cottrell, *et al.*, "Silence of the strands: RNA interference in eukaryotic pathogens." Trends Microbiol. 2003 Jan;11(1):37-15 43; Links, "Mammalian RNAi for the masses." Trends Genet. 2003 Jan;19(1):9-12; Hamada, *et al.*, "Effects on RNA interference in gene expression (RNAi) in cultured mammalian cells of mismatches and the introduction of chemical modifications at the 3'-ends of siRNAs." Antisense Nucleic Acid Drug Dev. 2002 Oct;12(5):301-9; Links, "RNAi and related mechanisms and their potential use for therapy." Curr. Opin. Chem. Biol. 2002 Dec;6(6):829-20 34; Kawasaki, *et al.*, "Short hairpin type of dsRNAs that are controlled by tRNA(Val) promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells." Nucleic Acids Res. 2003 Jan 15;31(2):700-7.)

25 A nick is two non-linked nucleomonomers in an oligonucleotide. A nick can be included at any point along the sense or antisense nucleotide sequence. In a preferred embodiment, a nick is in the sense sequence. In another preferred embodiment, the nick is at least about four nucleomonomers in from an end of the duplexed region of the oligonucleotide (*e.g.*, is at least about four nucleomonomers away from the 5' or 3' end of the oligonucleotide or away from a loop structure. For example, in one embodiment, the nick is present in the middle of the sense strand of the duplex molecule (*e.g.*, if the sense sequence of 30 the duplex is 30 nucleomonomers in length, nucleomonomers 14 and 15 or 15 and 16 are unlinked). In an embodiment, a nick may optionally be ligated to form a circular nucleic acid 35 molecule.

For example, in the structure below, the indicated U nucleomonomer is not bonded to the neighboring nucleomonomer, *e.g.*, by a phosphodiester bond. The 5' OH of the nick may 35 optionally be phosphorylated to allow enzymatic ligation of the oligonucleotide into a circle.

*Figure 5.*

5

As used herein, the term "nucleotide" includes any monomeric unit of DNA or RNA containing a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is usually linked to the sugar moiety via the glycosidic carbon (at the 1' carbon of pentose) and that combination of base and sugar is called a "nucleoside." The base 10 characterizes the nucleotide with the four customary bases of DNA being adenine (A), guanine (G), cytosine (C) and thymine (T). Inosine (I) is an example of a synthetic base that can be used to substitute for any of the four, naturally-occurring bases (A, C, G, or T). The four RNA bases are A, G, C, and uracil (U). Accordingly, an oligonucleotide may be a 15 nucleotide sequence comprising a linear array of nucleotides connected by phosphodiester bonds between the 3' and 5' carbons of adjacent pentoses. Other modified nucleosides/nucleotides are described herein and may also be used in the oligonucleotides of the invention.

Oligonucleotides may comprise, for example, oligonucleotides, oligonucleosides, polydeoxyribonucleotides (containing 2'-deoxy-D-ribose) or modified forms thereof, e.g., 20 DNA, polyribonucleotides (containing D-ribose or modified forms thereof), RNA, or any other type of polynucleotide which is an N-glycoside or C-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base. The term oligonucleotide includes compositions in which adjacent nucleomonomers are linked via phosphorothioate, amide or other linkages (e.g., Neilsen, P.E., et al. 1991. *Science*. 254:1497). Generally, the term 25 "linkage" refers to any physical connection, preferably covalent coupling, between two or more nucleic acid components, e.g., catalyzed by an enzyme such as a ligase.

In addition to its art-recognized meaning (e.g., a relatively short length single or double-stranded sequences of deoxyribonucleotides or ribonucleotides linked via phosphodiester bonds), the term "oligonucleotide" includes any structure that serves as a 30 scaffold or support for the bases of the oligonucleotide, where the scaffold permits binding to the target nucleic acid molecule in a sequence-dependent manner.

Oligonucleotides of the invention are isolated. The term "isolated" includes nucleic acid molecules which are synthesized (*e.g.*, chemically, enzymatically, or recombinantly) or are naturally occurring but separated from other nucleic acid molecules which are present in a natural source of the nucleic acid. Preferably, a naturally occurring "isolated" nucleic acid molecule is free of sequences which naturally flank the nucleic acid molecule (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid molecule) in a nucleic acid molecule in an organism from which the nucleic acid molecule is derived.

- The term "nucleomonomer" includes a single base covalently linked to a second moiety. Nucleomonomers include, for example, nucleosides and nucleotides.
- Nucleomonomers can be linked to form oligonucleotides that bind to target nucleic acid sequences in a sequence specific manner.

In one embodiment, modified (non-naturally occurring) nucleomonomers can be used in the oligonucleotides described herein. For example, nucleomonomers which are based on bases (purines, pyrimidines, and derivatives and analogs thereof) bound to substituted and unsubstituted cycloalkyl moieties, *e.g.*, cyclohexyl or cyclopentyl moieties, and substituted and unsubstituted heterocyclic moieties, *e.g.*, 6-member morpholino moieties or, preferably, sugar moieties.

Sugar moieties include natural, unmodified sugars, *e.g.*, monosaccharides (such as pentoses, *e.g.*, ribose, deoxyribose), modified sugars and sugar analogs. Possible modifications of nucleomonomers, particularly of a sugar moiety, include, for example, replacement of one or more of the hydroxyl groups with a halogen, a heteroatom, an aliphatic group, or the functionalization of the hydroxyl group as an ether, an amine, a thiol, or the like. One particularly useful group of modified nucleomonomers are 2'-O-methyl nucleotides, especially when the 2'-O-methyl nucleotides are used as nucleomonomers in the ends of the oligomers. Such 2'O-methyl nucleotides may be referred to as "methylated," and the corresponding nucleotides may be made from unmethylated nucleotides followed by alkylation or directly from methylated nucleotide reagents. Modified nucleomonomers may be used in combination with unmodified nucleomonomers. For example, an oligonucleotide of the invention may contain both methylated and unmethylated nucleomonomers.

Some exemplary modified nucleomonomers include sugar- or backbone-modified ribonucleotides. Modified ribonucleotides may contain a nonnaturally occurring base (instead of a naturally occurring base) such as uridines or cytidines modified at the 5-position, *e.g.*, 5-(2-amino)propyl uridine and 5-bromo uridine; adenosines and guanosines modified at the 8-position, *e.g.*, 8-bromo guanosine; deaza nucleotides, *e.g.*, 7-deaza-adenosine; and N-alkylated nucleotides, *e.g.*, N6-methyl adenosine. Also, sugar-modified ribonucleotides may have the 2'-OH group replaced by a H, alxoxy (or OR), R or alkyl, halogen, SH, SR, amino (such as NH<sub>2</sub>, NHR, NR<sub>2</sub>), or CN group, wherein R is lower alkyl, alkenyl, or alkynyl.

Modified ribonucleotides may also have the phosphoester group connecting to adjacent ribonucleotides replaced by a modified group, *e.g.*, of phosphothioate group. More generally, the various nucleotide modifications may be combined.

In one embodiment, sense oligomers may have 2' modifications on the ends (1 on each end, 2 on each end, 3 on each end, and 4 on each end, and so on; as well as 1 on one end, 2 on one end, 3 on one end, and 4 on one end, and so on; and even unbalanced combinations such as 1 on one end and 2 on the other end, and so on). Likewise, the antisense strand may have 2' modifications on the ends (1 on each end, 2 on each end, 3 on each end, and 4 on each end, and so on; as well as 1 on one end, 2 on one end, 3 on one end, and 4 on one end, and so on; and even unbalanced combinations such as 1 on one end and 2 on the other end, and so on). In preferred aspects, such 2'-modifications are in the sense RNA strand or the sequences other than the antisense strand.

To further maximize endo- and exonuclease resistance, in addition to the use of 2' modified nucleomonomers in the ends, inter-nucleomonomer linkages other than phosphodiesters may be used. For example, such end blocks may be used alone or in conjunction with phosphothorothioate linkages between the 2'-O-methyl linkages. Preferred 2'-modified nucleomonomers are 2'-modified C and U bases.

Although the antisense strand may be substantially identical to at least a portion of the target gene (or genes), at least with respect to the base pairing properties, the sequence need not be perfectly identical to be useful, *e.g.*, to inhibit expression of a target gene's phenotype. Generally, higher homology can be used to compensate for the use of a shorter antisense gene. In some cases, the antisense strand generally will be substantially identical (although in antisense orientation) to the target gene.

One particular example of a composition of the invention has end-blocks on both ends of a sense oligonucleotide and only the 3' end of an antisense oligonucleotide. Without wishing to be bound by theory, the inventors believe that a 2'-O-modified sense strand works less well than unmodified because it is not efficiently unwound. Accordingly, another 5 embodiment of the invention includes duplexes in which nucleomonomer-nucleomonomer mismatches are present in a sense 2'-O-methyl strand (and are thought to be easier to unwind).

Accordingly, for a given first oligonucleotide strand, a number of complementary 10 second oligonucleotide strands are permitted according to the invention. For example, in the following Tables, a targeted and a non-targeted oligonucleotide are illustrated with several possible complementary oligonucleotides. The individual nucleotides may be 2'-OH RNA nucleotides (R) or the corresponding 2'-OMe nucleotides (M), and the oligonucleotides themselves may contain mismatched nucleotides (lower case letters).

15 Targeted Oligonucleotide:

First Strand:	CCCUUCUGUCUUGAACAUAGAG	(SEQ ID NO: ##)
Second Strand:	CTgATGTTCAAGACAGAACGG	(SEQ ID NO: ##)
(methyl groups →)	MMMMMMMMMMMMMMMMMMMM	
	CTgATGTTCAAGACAGAACGG	(SEQ ID NO: ##)
	RRRRRRRRRRRRRRRRRDD	
	CTCAUGUUCAAGACAGAACGG	(SEQ ID NO: ##)
	RRRRRRMMMMMMMMRRRRRR	
	CTCAUGUUCAAGACAGAACGG	(SEQ ID NO: ##)
	MMMMMMRRRRRRRRMMMM	
	CTCAUGUUCAAGACAGAACGG	(SEQ ID NO: ##)
	RMRMRMRMRRMRRMRRM	

### *Non-Targeted Oligonucleotide:*

First Strand:	GAGTACAAGTTCTGTCTTCCC	(SEQ ID NO: ##)
Second Strand:	GGcAAGACAGAACTTGTAgTC	(SEQ ID NO: ##)
(methyl groups →)	MMMMMMMMMMMMMMMMMMMM	
	GGGAAGACAGAACTTGTACTC	(SEQ ID NO: ##)
	RRRRRRMmmmmmmmmRRRRRR	
	GGGAAGACAGAACTTGTACTC	(SEQ ID NO: ##)
	MMmmmmRrrrrrrRmmmmmm	
	GGGAAGACAGAACTTGTACTC	(SEQ ID NO: ##)
	RMRMrmRMrmRMrmRMrmR	

Another example of further modifications that may be used in conjunction with 2'-O-methyl nucleomonomers are modification of the sugar residues themselves, for example alternating modified and unmodified sugars, particularly in the sense strand.

In some embodiments, the length of the sense strand can be 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, or 18 nucleotides, with a complementary duplexed RNA strand, optionally having overhangs.

As a further example, the use of 2'-O-methyl RNA may beneficially be used in circumstances in which it is desirable to minimize cellular stress responses. RNA having 2'-O-methyl nucleomonomers may not be recognized by cellular machinery that is thought to recognize unmodified RNA. The use of 2'-O-methylated or partially 2'-O-methylated RNA may avoid the interferon response to double-stranded nucleic acids, while maintaining target RNA inhibition. This RNAi (“stealth RNAi”) is useful for avoiding the interferon or other cellular stress responses, both in short RNAi (*e.g.*, siRNA) sequences that induce the interferon response, and in longer RNAi sequences that may induce the interferon response.

An especially advantageous use of the present invention is in gene function studies in which multiple RNAi sequences are used. According to present methods known in the art, frequently there is no way of predicting which sequences might induce a stress response, including the interferon response, and in this regard the present invention significantly advances the state of the art. For example, if all of the multiple sequences are partially 2'-O-methylated, the stress response, including interferon response, may be avoided, and thus avoid confounding results in which some sequences affect cellular phenotype independent of the target gene inhibition. Other chemical modifications in addition to 2'-O-methylation may also achieve this effect.

For example, modified sugars include D-ribose, 2'-O-alkyl (including 2'-O-methyl and 2'-O-ethyl), *i.e.*, 2'-alkoxy, 2'-amino, 2'-S-alkyl, 2'-halo (including 2'-fluoro), 2'-methoxyethoxy, 2'-allyloxy (-OCH<sub>2</sub>CH=CH<sub>2</sub>), 2'-propargyl, 2'-propyl, ethynyl, ethenyl, propenyl, and cyano and the like. In one embodiment, the sugar moiety can be a hexose and incorporated into an oligonucleotide as described (Augustyns, K., *et al.*, *Nucl. Acids. Res.* 1992, 18:4711). Exemplary nucleomonomers can be found, *e.g.*, in U.S. Patent 5,849,902, incorporated by reference herein.

The term “alkyl” includes saturated aliphatic groups, including straight-chain alkyl groups (*e.g.*, methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, *etc.*), branched-chain alkyl groups (isopropyl, tert-butyl, isobutyl, *etc.*), cycloalkyl (alicyclic) groups (cyclopropyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl), alkyl substituted cycloalkyl groups, and cycloalkyl substituted alkyl groups. In certain embodiments, a straight chain or branched chain alkyl has 6 or fewer carbon atoms in its backbone (*e.g.*, C<sub>1</sub>-C<sub>6</sub> for straight chain, C<sub>3</sub>-C<sub>6</sub> for branched chain), and more preferably 4 or fewer. Likewise, preferred cycloalkyls have from 3-8 carbon atoms in their ring structure, and more preferably have 5 or 6 carbons in the ring structure. The term C<sub>1</sub>-C<sub>6</sub> includes alkyl groups containing 1 to 6 carbon atoms.

Moreover, unless otherwise specified, the term alkyl includes both “unsubstituted alkyls” and “substituted alkyls,” the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, alkenyl, alkynyl, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxy carbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxy carbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxy, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulphydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety. Cycloalkyls can be further substituted, *e.g.*, with the substituents described above. An “alkylaryl” or an “arylalkyl” moiety is an alkyl substituted with an aryl (*e.g.*, phenylmethyl (benzyl)). The term “alkyl” also includes the side chains of natural and unnatural amino acids. The term “n-alkyl” means a straight chain (*i.e.*, unbranched) unsubstituted alkyl group.

The term “alkenyl” includes unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double bond. For example, the term “alkenyl” includes straight-chain alkenyl groups (*e.g.*, ethylenyl, propenyl, butenyl, pentenyl, hexenyl, heptenyl, octenyl, nonenyl, decenyl, *etc.*), branched-chain alkenyl groups, cycloalkenyl (alicyclic) groups (cyclopropenyl, cyclopentenyl, cyclohexenyl, cycloheptenyl, cyclooctenyl), alkyl or alkenyl substituted cycloalkenyl groups, and cycloalkyl or cycloalkenyl substituted alkenyl groups. In certain embodiments, a straight chain or branched chain alkenyl group has 6 or fewer carbon atoms in its backbone (*e.g.*, C<sub>2</sub>-C<sub>6</sub> for straight chain, C<sub>3</sub>-C<sub>6</sub> for branched chain). Likewise, cycloalkenyl groups may have from 3-8 carbon atoms in their ring structure, and more preferably have 5 or 6 carbons in the ring structure. The term C<sub>2</sub>-C<sub>6</sub> includes alkenyl groups containing 2 to 6 carbon atoms.

Moreover, unless otherwise specified, the term alkenyl includes both “unsubstituted alkenyls” and “substituted alkenyls,” the latter of which refers to alkenyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, alkyl groups, alkynyl groups, halogens, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxy carbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxy carbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxy, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety.

The term “alkynyl” includes unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but which contain at least one triple bond. For example, the term “alkynyl” includes straight-chain alkynyl groups (*e.g.*, ethynyl, propynyl, butynyl, pentynyl, hexynyl, heptynyl, octynyl, nonynyl, decynyl, *etc.*), branched-chain alkynyl groups, and cycloalkyl or cycloalkenyl substituted alkynyl groups. In certain embodiments, a straight chain or branched chain alkynyl group has 6 or fewer carbon atoms in its backbone (*e.g.*, C<sub>2</sub>-C<sub>6</sub> for straight chain, C<sub>3</sub>-C<sub>6</sub> for branched chain). The term C<sub>2</sub>-C<sub>6</sub> includes alkynyl groups containing 2 to 6 carbon atoms.

Moreover, unless otherwise specified, the term alkynyl includes both “unsubstituted alkynyls” and “substituted alkynyls,” the latter of which refers to alkynyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, alkyl groups, alkynyl groups, halogens, hydroxyl, 5 alkylcarbonyloxy, arylcarbonyloxy, alkoxy carbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxy carbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, 10 alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety.

Unless the number of carbons is otherwise specified, “lower alkyl” as used herein means an alkyl group, as defined above, but having from one to five carbon atoms in its 15 backbone structure. “Lower alkenyl” and “lower alkynyl” have chain lengths of, for example, 2-5 carbon atoms.

The term “alkoxy” includes substituted and unsubstituted alkyl, alkenyl, and alkynyl groups covalently linked to an oxygen atom. Examples of alkoxy groups include methoxy, ethoxy, isopropoxy, propoxy, butoxy, and pentoxy groups. Examples of substituted alkoxy 20 groups include halogenated alkoxy groups. The alkoxy groups can be substituted with groups such as alkenyl, alkynyl, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxy carbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including 25 alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or 30 heteroaromatic moieties. Examples of halogen substituted alkoxy groups include, but are not limited to, fluoromethoxy, difluoromethoxy, trifluoromethoxy, chloromethoxy, dichloromethoxy, trichloromethoxy, etc.

The term “heteroatom” includes atoms of any element other than carbon or hydrogen. Preferred heteroatoms are nitrogen, oxygen, sulfur and phosphorus.

The term “hydroxy” or “hydroxyl” includes groups with an –OH or –O<sup>-</sup> (with an 35 appropriate counterion).

The term "halogen" includes fluorine, bromine, chlorine, iodine, *etc.* The term "perhalogenated" generally refers to a moiety wherein all hydrogens are replaced by halogen atoms.

The term "substituted" includes substituents which can be placed on the moiety and which allow the molecule to perform its intended function. Examples of substituents include 5 alkyl, alkenyl, alkynyl, aryl,  $(CR'R'')_{0-3}NR'R''$ ,  $(CR'R'')_{0-3}CN$ ,  $NO_2$ , halogen,  $(CR'R'')_{0-3}C(halogen)_3$ ,  $(CR'R'')_{0-3}CH(halogen)_2$ ,  $(CR'R'')_{0-3}CH_2(halogen)$ ,  $(CR'R'')_{0-3}CONR'R''$ ,  $(CR'R'')_{0-3}S(O)_{1-2}NR'R''$ ,  $(CR'R'')_{0-3}CHO$ ,  $(CR'R'')_{0-3}O(CR'R'')_{0-3}H$ ,  $(CR'R'')_{0-3}S(O)_{0-2}R'$ ,  $(CR'R'')_{0-3}O(CR'R'')_{0-3}H$ ,  $(CR'R'')_{0-3}COR'$ ,  $(CR'R'')_{0-3}CO_2R'$ , or 10  $(CR'R'')_{0-3}OR'$  groups; wherein each R' and R'' are each independently hydrogen, a  $C_1-C_5$  alkyl,  $C_2-C_5$  alkenyl,  $C_2-C_5$  alkynyl, or aryl group, or R' and R'' taken together are a benzylidene group or a  $-(CH_2)_2O(CH_2)_2-$  group.

The term "amine" or "amino" includes compounds or moieties in which a nitrogen atom is covalently bonded to at least one carbon or heteroatom. The term "alkyl amino" 15 includes groups and compounds wherein the nitrogen is bound to at least one additional alkyl group. The term "dialkyl amino" includes groups wherein the nitrogen atom is bound to at least two additional alkyl groups.

The term "ether" includes compounds or moieties which contain an oxygen bonded to two different carbon atoms or heteroatoms. For example, the term includes "alkoxyalkyl," 20 which refers to an alkyl, alkenyl, or alkynyl group covalently bonded to an oxygen atom which is covalently bonded to another alkyl group.

The term "base" includes the known purine and pyrimidine heterocyclic bases, deazapurines, and analogs (including heterocyclic substituted analogs, *e.g.*, aminoethoxy phenoxazine), derivatives (*e.g.*, 1-alkyl-, 1-alkenyl-, heteroaromatic- and 1-alkynyl 25 derivatives) and tautomers thereof. Examples of purines include adenine, guanine, inosine, diaminopurine, and xanthine and analogs (*e.g.*, 8-oxo- $N^6$ -methyladenine or 7-diazaxanthine) and derivatives thereof. Pyrimidines include, for example, thymine, uracil, and cytosine, and their analogs (*e.g.*, 5-methylcytosine, 5-methyluracil, 5-(1-propynyl)uracil, 5-(1-propynyl)cytosine and 4,4-ethanocytosine). Other examples of suitable bases include non-purinyl and non-pyrimidinyl bases such as 2-aminopyridine and triazines. 30

In a preferred embodiment, the nucleomonomers of an oligonucleotide of the invention are RNA nucleotides. In another preferred embodiment, the nucleomonomers of an oligonucleotide of the invention are modified RNA nucleotides.

The term "nucleoside" includes bases which are covalently attached to a sugar moiety, preferably ribose or deoxyribose. Examples of preferred nucleosides include ribonucleosides and deoxyribonucleosides. Nucleosides also include bases linked to amino acids or amino acid analogs which may comprise free carboxyl groups, free amino groups, or 5 protecting groups. Suitable protecting groups are well known in the art (see P.G.M. Wuts and T.W. Greene, "Protective Groups in Organic Synthesis", 2<sup>nd</sup> Ed., Wiley-Interscience, New York, 1999).

The term "nucleotide" includes nucleosides which further comprise a phosphate group or a phosphate analog.

10 As used herein, the term "linkage" includes a naturally occurring, unmodified phosphodiester moiety (-O-(PO<sub>2</sub>)-O-) that covalently couples adjacent nucleomonomers. As used herein, the term "substitute linkage" includes any analog or derivative of the native phosphodiester group that covalently couples adjacent nucleomonomers. Substitute linkages include phosphodiester analogs, e.g., phosphorothioate, phosphorodithioate, and P-15 ethyoxyphosphodiester, P-ethoxyphosphodiester, P-alkyloxyphosphotriester, methylphosphonate, and nonphosphorus containing linkages, e.g., acetals and amides. Such substitute linkages are known in the art (e.g., Bjergarde *et al.* 1991. Nucleic Acids Res. 19:5843; Caruthers *et al.* 1991. Nucleosides Nucleotides. 10:47).

20 In certain embodiments, oligonucleotides of the invention comprise 3' and 5' termini (except for circular oligonucleotides). In one embodiment, the 3' and 5' termini of an oligonucleotide can be substantially protected from nucleases e.g., by modifying the 3' or 5' linkages (e.g., U.S. patent 5,849,902 and WO 98/13526). For example, oligonucleotides can be made resistant by the inclusion of a "blocking group." The term "blocking group" as used herein refers to substituents (e.g., other than OH groups) that can be attached to 25 oligonucleotides or nucleomonomers, either as protecting groups or coupling groups for synthesis (e.g., FITC, propyl (CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>3</sub>), phosphate (PO<sub>3</sub><sup>2-</sup>), hydrogen phosphonate, or phosphoramidite). "Blocking groups" also include "end blocking groups" or "exonuclease blocking groups" which protect the 5' and 3' termini of the oligonucleotide, including modified nucleotides and non-nucleotide exonuclease resistant structures.

Exemplary end-blocking groups include cap structures (e.g., a 7-methylguanosine cap), inverted nucleomonomers, e.g., with 3'-3' or 5'-5' end inversions (see, e.g., Ortiagao *et al.* 1992. *Antisense Res. Dev.* 2:129), methylphosphonate, phosphoramidite, non-nucleotide groups (e.g., non-nucleotide linkers, amino linkers, conjugates) and the like. The 3' terminal nucleomonomer can comprise a modified sugar moiety. The 3' terminal nucleomonomer comprises a 3'-O that can optionally be substituted by a blocking group that prevents 3'-exonuclease degradation of the oligonucleotide. For example, the 3'-hydroxyl can be esterified to a nucleotide through a 3'→3' internucleotide linkage. For example, the alkyloxy radical can be methoxy, ethoxy, or isopropoxy, and preferably, ethoxy. Optionally, the 3'→3' linked nucleotide at the 3' terminus can be linked by a substitute linkage. To reduce nuclease degradation, the 5' most 3'→5' linkage can be a modified linkage, e.g., a phosphorothioate or a P-alkyloxyphosphotriester linkage. Preferably, the two 5' most 3'→5' linkages are modified linkages. Optionally, the 5' terminal hydroxy moiety can be esterified with a phosphorus containing moiety, e.g., phosphate, phosphorothioate, or P-ethoxyphosphate.

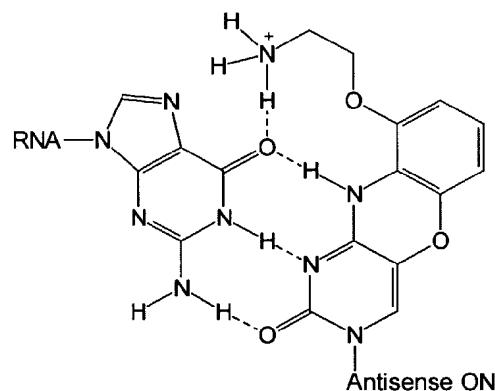
In one embodiment, the sense strand of an oligonucleotide comprises a 5' group that allows for RNAi activity but which renders the sense strand inactive in terms of gene targeting. Preferably, such a 5' modifying group is a phosphate group or a group larger than a phosphate group

In another embodiment, the antisense strand of an oligonucleotide comprises a 5' phosphate group.

In one embodiment, the oligonucleotides included in the composition are high affinity oligonucleotides. The term "high affinity" as used herein includes oligonucleotides that have a Tm (melting temperature) of or greater than about 60°C, greater than about 65°C, greater than about 70°C, greater than about 75°C, greater than about 80 °C or greater than about 85 °C. The Tm is the midpoint of the temperature range over which the oligonucleotide separates from the target nucleotide sequence. At this temperature, 50% helical (hybridized) versus coil (unhybridized) forms are present. Tm is measured by using the UV spectrum to determine the formation and breakdown (melting) of hybridization. Base stacking occurs during hybridization, which leads to a reduction in UV absorption. Tm depends both on GC content of the two nucleic acid molecules and on the degree of sequence complementarity. Tm can be determined using techniques that are known in the art (see for example, Monia *et al.* 1993. *J. Biol. Chem.* 268:145; Chiang *et al.* 1991. *J. Biol. Chem.* 266:18162; Gagnor *et al.* 1987. *Nucleic Acids Res.* 15:10419; Monia *et al.* 1996. *Proc. Natl. Acad. Sci.* 93:15481; Publisis and Tinoco. 1989. *Methods in Enzymology* 180:304; Thuong *et al.* 1987. *Proc. Natl. Acad. Sci. USA* 84:5129).

In one embodiment, an oligonucleotide can include an agent which increases the affinity of the oligonucleotide for its target sequence. The term "affinity enhancing agent" includes agents that increase the affinity of an oligonucleotide for its target. Such agents include, *e.g.*, intercalating agents and high affinity nucleomonomers. Intercalating agents interact strongly and nonspecifically with nucleic acids. Intercalating agents serve to stabilize RNA-DNA duplexes and thus increase the affinity of the oligonucleotides for their targets. Intercalating agents are most commonly linked to the 3' or 5' end of oligonucleotides. Examples of intercalating agents include acridine, chlorambucil, benzopyridoquinoxaline, benzopyridoindole, benzophenanthridine, and phenazinium. The agents may also impart other characteristics to the oligonucleotide, for example, increasing resistance to endonucleases and exonucleases.

In one embodiment, a high affinity nucleomonomer is incorporated into an oligonucleotide. The language "high affinity nucleomonomer" as used herein includes modified bases or base analogs that bind to a complementary base in a target nucleic acid molecule with higher affinity than an unmodified base, for example, by having more energetically favorable interactions with the complementary base, *e.g.*, by forming more hydrogen bonds with the complementary base. For example, high affinity nucleomonomer analogs such as aminoethoxy phenoxyazine (also referred to as a G clamp), which forms four hydrogen bonds with guanine are included in the term "high affinity nucleomonomer." A high affinity nucleomonomer is illustrated below (*see, e.g.*, Flanagan, *et al.*, 1999. *Proc. Natl. Acad. Sci.* 96:3513).



*Figure 6.*

Other exemplary high affinity nucleomonomers are known in the art and include 7-alkenyl, 7-alkynyl, 7-heteroaromatic-, or 7-alkynyl-heteroaromatic-substituted bases or the like which can be substituted for adenosine or guanosine in oligonucleotides (see, e.g., U.S. patent 5,594,121). Also, 7-substituted deazapurines have been found to impart enhanced binding properties to oligonucleotides, i.e., by allowing them to bind with higher affinity to complementary target nucleic acid molecules as compared to unmodified oligonucleotides. High affinity nucleomonomers can be incorporated into the oligonucleotides of the instant invention using standard techniques.

In another embodiment, an agent that increases the affinity of an oligonucleotide for its target comprises an intercalating agent. As used herein, the language "intercalating agent" includes agents which can bind to a DNA double helix. When covalently attached to an oligonucleotide of the invention, an intercalating agent enhances the binding of the oligonucleotide to its complementary genomic DNA target sequence. The intercalating agent may also increase resistance to endonucleases and exonucleases.

Exemplary intercalating agents are taught by Helene and Thuong (1989. *Genome* 31:413), and include e.g., acridine derivatives (Lacoste *et al.* 1997. *Nucleic Acids Research*. 25:1991; Kukreti *et al.* 1997. *Nucleic Acids Research*. 25:4264); quinoline derivatives (Wilson *et al.* 1993. *Biochemistry* 32:10614); benzo[f]quino[3,4-b]quinoxaline derivatives (Marchand *et al.* 1996. *Biochemistry*. 35:5022; Escude *et al.* 1998. *Proc. Natl. Acad. Sci.* 95:3591).

Intercalating agents can be incorporated into an oligonucleotide using any convenient linkage. For example, acridine or psoralen can be linked to the oligonucleotide through any available -OH or -SH group, e.g., at the terminal 5' position of the oligonucleotide, the 2' positions of sugar moieties, or an OH, NH<sub>2</sub>, COOH, or SH incorporated into the 5-position of pyrimidines using standard methods.

In one embodiment, when included in an RNase H activating antisense nucleotide sequence, an agent that increases the affinity of an oligonucleotide for its target is not positioned adjacent to an RNase activating region of the oligonucleotide, *e.g.*, is positioned adjacent to a non-RNase activating region. Preferably, the agent that increases the affinity of an oligonucleotide for its target is placed at a distance as far as possible from the RNase activating domain of the chimeric antisense sequence such that the specificity of the chimeric antisense sequence is not altered when compared with the specificity of a chimeric antisense sequence which lacks the intercalating compound. In one embodiment, this can be accomplished by positioning the agent adjacent to a non-RNase activating region. The specificity of the oligonucleotide can be tested by demonstrating that transcription of a non-target sequence, preferably a non-target sequence which is structurally similar to the target (*e.g.*, has some sequence homology or identity with the target sequence but which is not identical in sequence to the target), is not inhibited to a greater degree by an oligonucleotide comprising an affinity enhancing agent than by an oligonucleotide directed against the same target that does not comprise an affinity enhancing agent.

The double-stranded oligonucleotides of the invention may be formed by a single, self-complementary nucleic acid strand or two separate complementary nucleic acid strands. Duplex formation can occur either inside or outside the cell containing the target gene.

As used herein, the term "double-stranded" includes one or more nucleic acid molecules comprising a region of the molecule in which at least a portion of the nucleomonomers are complementary and hydrogen bond to form a duplex.

As used herein, the term "duplex" includes the region of the double-stranded nucleic acid molecule(s) that is (are) hydrogen bonded to a complementary sequence.

The double-stranded oligonucleotides of the invention comprise a nucleotide sequence that is sense to a target gene and a complementary sequence that is antisense to the target gene. The sense and antisense nucleotide sequences correspond to the target gene sequence, *e.g.*, are identical or are sufficiently identical to effect target gene inhibition (*e.g.*, are about at least about 98%, 96% identical, 94%, 90% identical, 85% identical, or 80% identical) to the target gene sequence.

When comprised of two separate complementary nucleic acid molecules, the individual nucleic acid molecules can be of different lengths.

In one embodiment, a double-stranded oligonucleotide of the invention is double-stranded over its entire length, *i.e.*, with no overhanging single-stranded sequence at either end of the molecule, *i.e.*, is blunt-ended. In another embodiment, a double-stranded oligonucleotide of the invention is not double-stranded over its entire length. For instance,

5 when two separate nucleic acid molecules are used, one of the molecules, *e.g.*, the first molecule comprising an antisense sequence can be longer than the second molecule hybridizing thereto (leaving a portion of the molecule single-stranded). Likewise, when a single nucleic acid molecule is used a portion of the molecule at either end can remain single-stranded.

10 In one embodiment, a double-stranded oligonucleotide of the invention is double-stranded over at least about 70% of the length of the oligonucleotide. In another embodiment, a double-stranded oligonucleotide of the invention is double-stranded over at least about 80% of the length of the oligonucleotide. In another embodiment, a double-stranded oligonucleotide of the invention is double-stranded over at least about 90%-95% of the length of the oligonucleotide. In another embodiment, a double-stranded oligonucleotide of the invention is double-stranded over at least about 96%-98% of the length of the oligonucleotide.

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20 In one embodiment, the double-stranded duplex constructs of the invention can be further stabilized against nucleases by forming loop structures at the 5' or 3' end of the sense or antisense strand of the construct. For example, the construct can take the form:

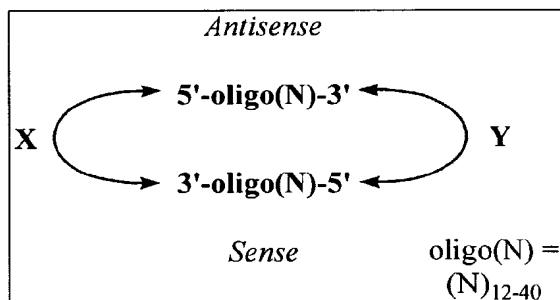


Figure 1.

where the Ns are nucleomonomers in complementary oligonucleotide strands (*i.e.*, the top N strand is complementary to the bottom N strand) of equal length (*e.g.*, between about 12 and about 40 nucleotides in length) and X and Y are each independently selected from a group consisting of nothing (*i.e.*, the construct is a blunt ended construct with no loops and no overhang); from about 1 to about 20 nucleotides of 5' overhang; from about 1 to about 20 nucleotides of 3' overhang; a GAAA loop (tetra-loop); and a loop consisting from about 4 to about 20 nucleomonomers (where the nucleomonomers are all either Gs or A's).

The sequence of Ns corresponds to the target gene sequence (*e.g.*, is homologous or identical to a nucleotide sequence that is sense or antisense to the target gene sequence), while the nucleotide sequence of the loop structure does not correspond to the target gene sequence.

For example, such loops can comprise all Gs and A's and be from about 4 to about 20 nucleotides in length. In one embodiment, such a loop can be a tetra-loop having a sequence GAAA:

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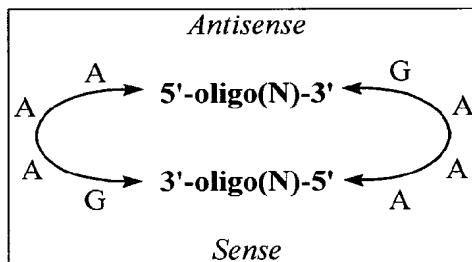
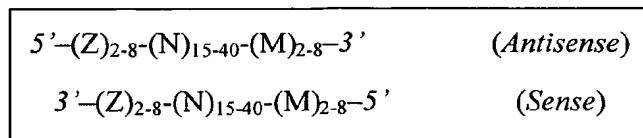


Figure 7.

In one embodiment, the number of Ns is about 27.

20 In embodiments in which loops are at one or both ends of the construct, the oligonucleotide can be divided by having a "nick" which is two non-linked nucleomonomers at any point along the sense or antisense strand, but preferably along the sense strand. Preferably, the nick is at least four bases from the nearest end of the duplexed region (to provide enough thermodynamic stability).

25 In another embodiment, a construct of the invention can take the form:

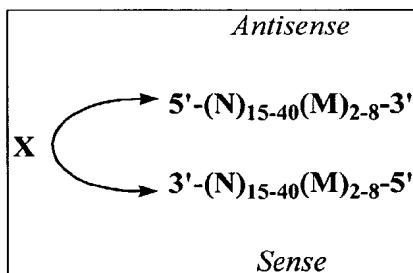
*Figure 8.*

- 5 where the Ns are complementary nucleomonomers in oligonucleotide strands of equal length (e.g., between 12-40 nucleomonomers in length); Zs are nucleomonomers in complementary oligonucleotide strands of between about 2 and about 8 nucleomonomers in length and which comprise a sequence which can optionally correspond to the target sequence; and where Ms are nucleomonomers in complementary oligonucleotide strands of between about 2 and about  
10 8 nucleomonomers in length and which can optionally correspond to the target sequence.

Preferably, the Zs and Ms are nucleomonomers selected from the group consisting of Cs and Gs to make the end of the duplex more thermodynamically stable. Ends of duplexes can become single stranded transiently, and since duplex RNA is more stable than single-stranded RNA, the enhanced stability of the duplex on the ends will result in higher nuclease  
15 stability.

A preferred sequence for Z or M in the antisense strand is from 2-8 nucleomonomers in length or preferably from 3-4 nucleomonomers in length, e.g., (from 5' to 3') CC, GG, CG, GC, CCC, GGG, CGG, GCC, GCG, CGC, CGGG, GCCC, CCCC, GGGG, GCGC, CGCG, GGGC, CCCG, CGGG, GCCC, GGCC, or CCGG. The complementary strand would have  
20 the corresponding complementary sequence.

In still another embodiment, a construct of the invention has the form:

*Figure 3.*

where Ns are nucleomonomers in complementary oligonucleotide strands (*i.e.*, the top N strand is complementary to the bottom N strand) of equal length (*e.g.*, from between about 12 to about 40 nucleomonomers in length) and X is selected from the group consisting of nothing (*i.e.*, leaving blunt ends with no loop or overhang); 1-20 nucleotides of 5' overhang; 5 1-20 nucleotides of 3' overhang; a GAAA loop (tetra-loop); and a loop consisting of from about 4 to about 20 nucleomonomers (where the nucleomonomers are all either Gs or A's) and where Ms are nucleomonomers in complementary oligonucleotide strands of between about 2 and about 8 nucleomonomers in length (which can optionally correspond to the target sequence). Preferably, Ms are nucleomonomers selected from the group consisting of contain 10 Cs and Gs.

A preferred sequence for M in the antisense strand is from 2-8 nucleomonomers in length or preferably from 3-4 nucleomonomers in length, *e.g.*, (from 5' to 3') CC, GG, CG, GC, CCC, GGG, CGG, GCC, GCG, CGC, CGGG, GCCC, CCCC, GGGG, GCGC, CGCG, GGGC, CCCG, CGGG, GCCC, GGCC, or CCGG and the corresponding complement on the 15 opposite strand.

In another embodiment, the construct can take the form:

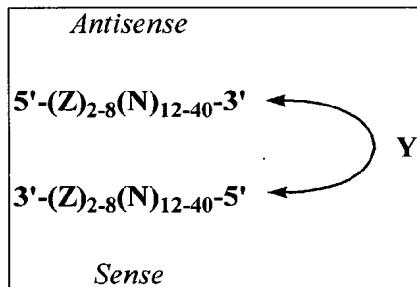
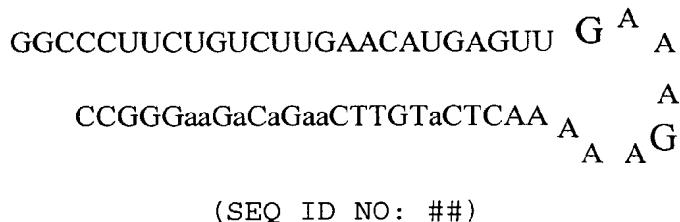


Figure 4.

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where Ns are nucleomonomers in complementary oligonucleotide strands of equal length (*e.g.*, from between about 12 to about 40 nucleomonomers in length) and Y is selected from the group consisting of nothing (*i.e.*, leaving blunt ends with no loop or overhang; 1-20 25 nucleotides of 5' overhang; 1-20 nucleotides of 3' overhang; a GAAA loop (tetra-loop); and a loop consisting of a sequence of from about 4 to about 20 nucleomonomers (where the nucleomonomers are all either Gs or A's) and where Zs are nucleomonomers in complementary oligonucleotide strands of between about 2 and about 8 nucleomonomers in length and which comprise a sequence which can optionally correspond to the target sequence. Preferably, the Zs are nucleomonomers selected from the group consisting of Cs 30 and Gs to make the end of the duplex more stable.

A preferred sequence for Z in the antisense strand is from 2-8 nucleomonomers in length or preferably from 3-4 nucleomonomers in length, e.g., (from 5' to 3') CC, GG, CG, GC, CCC, GGG, CGG, GCC, GCG, CGC, CGGG, GCCC, CCCC, GGGG, GCGC, CGCG, GGGC, CCCG, CGGG, GCCC, GGCC or CCGG (and the corresponding complement on the opposite strand). For example, in the following structure, GGCC on the end (and its complement) confers additional stability:

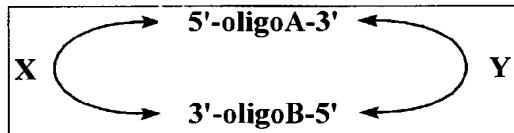


10

*Figure 9.*

The invention also relates to a double-stranded oligonucleotide composition having the following structure:

15

*Figure 10.*

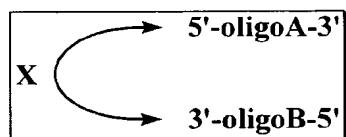
wherein (1) oligoA is an oligonucleotide of a number of nucleomonomers; (2) oligoB is an oligonucleotide that has the same number of nucleomonomers as oligoA and that is complementary to oligoA; (3) either oligoA or oligoB corresponds to a target gene sequence.

In this structure, X may be selected from (a) nothing; (b) an oligonucleotide of about 1 to about 20 nucleotides covalently bonded to the 5' end of oligoA and constituting a 5' overhang; (c) an oligonucleotide of about 1 to about 20 nucleotides covalently bonded to the 3' end of oligoB and constituting a 3' overhang; (d) and an oligonucleotide of about 4 to about 20 nucleomonomers covalently bonded to the 3' end of oligoB and the 5' end of oligoA and constituting a loop structure, where the nucleomonomers are selected from the group consisting of G and A.

Similarly, Y may be selected from (a) nothing; (b) an oligonucleotide of about 1 to about 20 nucleotides covalently bonded to the 5' end of oligoB and constituting a 5' overhang; (c) an oligonucleotide of about 1 to about 20 nucleotides covalently bonded to the 3' end of oligoA and constituting a 3' overhang; (d) and an oligonucleotide of about 4 to 5 about 20 nucleomonomers covalently bonded to the 3' end of oligoA and the 5' end of oligoB and constituting a loop structure, where the nucleomonomers are selected from the group consisting of G and A.

Similarly, the invention includes a double-stranded oligonucleotide composition having the structure:

10

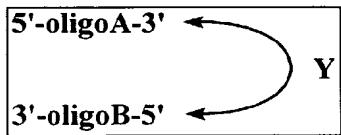


*Figure 11.*

wherein (1) oligoA is 5'-(N)<sub>15-40</sub>-(M)<sub>2-8</sub>-3' and oligoB is 5'-(N)<sub>15-40</sub>-(M)<sub>2-8</sub>-3', wherein each of N and M is independently a nucleomonomer; (2) both of the sequences of Ns are complementary oligonucleotide strands of equal length having between about 15 and 40 nucleomonomers; (3) at least one of the sequences of Ns, optionally with some or all of the flanking Ms, corresponds to a target gene sequence. Both of the sequences of Ms are complementary oligonucleotide strands of between about 2 and about 8 nucleomonomers in length. The two M strands are optionally of the same length.

The group X indicated by the curved line is selected from (a) nothing; (b) an oligonucleotide of about 1 to about 20 nucleotides covalently bonded to the 5' end of oligoA and constituting a 5' overhang; (c) an oligonucleotide of about 1 to about 20 nucleotides covalently bonded to the 3' end of oligoB and constituting a 3' overhang; (d) and an oligonucleotide of about 4 to about 20 nucleomonomers covalently bonded to the 3' end of oligoB and the 5' end of oligoA and constituting a loop structure, where the nucleomonomers are selected from the group consisting of G and A.

Likewise, the invention pertains to a double-stranded oligonucleotide composition having the structure:



*Figure 12.*

5 wherein (1) oligoA is 5'-(Z)<sub>2-8</sub>-(N)<sub>12-40</sub>-3' and oligoB is 5'-(Z)<sub>2-8</sub>-(N)<sub>12-40</sub>-3', wherein each  
of N and Z is independently a nucleomonomer; (2) both of the sequences of Ns are  
complementary oligonucleotide strands of equal length having between about 12 and 40  
nucleomonomers; (3) at least one of the sequences of Ns, optionally with some or all of the  
flanking Zs, corresponds to a target gene sequence. Both of the sequences of Zs are  
10 complementary oligonucleotide strands of between about 2 and about 8 nucleomonomers in  
length. The two Z strands are optionally of the same length.

Here, Y is selected from (a) nothing; (b) an oligonucleotide of about 1 to about 20  
nucleotides covalently bonded to the 5' end of oligoB and constituting a 5' overhang; (c) an  
15 oligonucleotide of about 1 to about 20 nucleotides covalently bonded to the 3' end of oligoA  
and constituting a 3' overhang; (d) and an oligonucleotide of about 4 to about 20  
nucleomonomers covalently bonded to the 3' end of oligoA and the 5' end of oligoB and  
consisting of G and A.

In one embodiment, the double-stranded duplex of an oligonucleotide of the invention  
20 is from between about 12 to about 50 nucleomonomers in length, *i.e.*, the number of  
nucleotides of the double-stranded oligonucleotide which hybridize to the complementary  
sequence of the double-stranded oligonucleotide to form the double-stranded duplex structure  
is from about 12 to about 50 nucleomonomers in length. In another embodiment, the double-  
stranded duplex of an oligonucleotide of the invention is from between about 12 to about 40  
25 nucleomonomers in length.

In one embodiment, the double-stranded duplex of an oligonucleotide of the  
invention is at least about 25 nucleomonomers in length. In one embodiment, the double-  
stranded duplex is greater than about 25 nucleomonomers in length. In one embodiment, a  
30 double-stranded duplex is at least about 26, 27, 28, 29, 30, at least about 40, at least about 50,  
or at least about 60, at least about 70, at least about 80, or at least about 90 nucleomonomers  
in length. In another embodiment, the double-stranded duplex is less than about 25  
nucleomonomers in length. In one embodiment, a double-stranded duplex is at least about  
10, at least about 15, at least about 20, at least about 22, at least about 23 or at least about 24  
nucleomonomers in length.

In one embodiment, the number of Ns in each strand of the duplex is about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, or 27. In another embodiment, the number of Ns in each strand of the duplex is about 30, 35, 40, 45, or 50. In one embodiment, the number of Ns in each strand of the duplex is about 19. In a preferred embodiment, the number of Ns in each strand of the duplex is about 27. In another embodiment, the number of Ns in each strand of the duplex is about 27 (*e.g.*, is 26, 27, or 28). In another embodiment, the number of Ns in each strand of the duplex is 27.

In one embodiment, an individual nucleic acid molecule of a double-stranded oligonucleotide of the invention is at least about 25 nucleomonomers in length. For example, when the double-stranded oligonucleotide of the invention is comprised of one nucleic acid molecule, that individual molecule is at least about 25 nucleomonomers in length or when the double-stranded oligonucleotide of the invention is comprised of two separate nucleic acid molecules, the length of at least one of the individual nucleic acid molecules is at least about 25 nucleomonomers in length.

A variety of nucleotides of different lengths may be used. In one embodiment, an individual nucleic acid molecule comprising a double-stranded oligonucleotide of the invention is greater than about 25 nucleomonomers in length. In one embodiment, an individual nucleic acid molecule comprising a double-stranded oligonucleotide of the invention is at least about 26, 27, 28, 29, 30, at least about 40, at least about 50, or at least about 60, at least about 70, at least about 80, or at least about 90 nucleomonomers in length. In another embodiment, an individual nucleic acid molecule comprising a double-stranded oligonucleotide of the invention is less than about 25 nucleomonomers in length. In one embodiment, an individual nucleic acid molecule comprising a double-stranded oligonucleotide of the invention is at least about 10, at least about 15, at least about 20, at least about 22, at least about 23 or at least about 24 nucleomonomers in length.

The double-stranded molecules of the invention comprise a first nucleotide sequence which is antisense to at least part of the target gene and a second nucleotide sequence which is complementary to the first nucleotide sequence; *i.e.*, is sense to at least part of the target gene. In one embodiment, the second nucleotide sequence of the double-stranded molecule comprises a nucleotide sequence which is at least about 100 % complementary to the antisense molecule.

In another embodiment, the second nucleotide sequence of the double-stranded molecule comprises a nucleotide sequence which is at least about 95 % complementary to the antisense molecule. In another embodiment, the second nucleotide sequence of the double-stranded molecule comprises a nucleotide sequence which is at least about 90 %

5 complementary to the antisense molecule. In another embodiment, the second nucleotide sequence of the double-stranded molecule comprises a nucleotide sequence which is at least about 80 % complementary to the antisense molecule. In another embodiment, the second nucleotide sequence of the double-stranded molecule comprises a nucleotide sequence which is at least about 60 % complementary to the antisense molecule. In another embodiment, the

10 second nucleotide sequence of the double-stranded molecule comprises a nucleotide sequence which is at least about 100 % complementary to the antisense molecule.

To determine the percent identity of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-identical sequences can be disregarded for comparison purposes). When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be

15 introduced for optimal alignment of the two sequences. The percent complementarity can be determined analogously; when a position in one sequence occupied by a nucleotide that is complementary to the nucleotide in the other sequence, then the molecules are

20 complementary at that position.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two nucleotide sequences is determined using e.g., the GAP program in the GCG software package, using a NWSgapdna. CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two nucleotide sequences is determined using the algorithm of E.

25 Meyers and W. Miller (*Comput. Appl. Biosci.* 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length

30 penalty of 12 and a gap penalty of 4.

The nucleic acid sequences of the present invention can further be used as a “query sequence” to perform alignments against sequences in public databases. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the  
5 NBLAST program, score = 100, wordlength = 12. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. *See, e.g.*, the NIH website.

10 In yet another embodiment, a first antisense sequence of the double-stranded molecule hybridizes to its complementary second sequence of the double-stranded molecule under stringent hybridization conditions. As used herein, the term “hybridizes under stringent conditions” is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 60% complementary to each other typically remain hybridized  
15 to each other. Preferably, the conditions are such that sequences at least about 70%, more preferably at least about 80%, even more preferably at least about 85% or 90% complementary to each other typically remain hybridized to each other.

Such stringent conditions are known to those skilled in the art and can be found in  
20 *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50°C, preferably at 55°C, more preferably at 60°C, and even more preferably at 65°C. Ranges intermediate to the above-recited values, *e.g.*, at 60-65°C or at 25 55-60°C are also intended to be encompassed by the present invention. Alternatively, formamide can be included in the hybridization solution, using methods and conditions also known in the art.

One of the sequences (or molecules) of the double-stranded oligonucleotide of the invention is antisense to the target gene. As used herein, the term “antisense sequence” includes nucleotide sequences which bind to the “sense” strand of the nucleotide sequence of  
30 the target gene (*e.g.*, polynucleotides such as DNA, mRNA (including pre-mRNA)) molecules. When the antisense sequences of the invention bind to nucleic acid molecules, they can bind to any region of a nucleic acid molecule, including *e.g.*, introns, exons, 5’, or 3’ untranslated regions. Antisense sequences that work by binding to a target and activating RNase H preferably bind within an intron, an exon, the 5’ untranslated region, or the 3’  
35 untranslated region of a nucleic acid target molecule.

Preferably, the oligonucleotide compositions of the invention do not activate the interferon pathway, *e.g.*, as evidenced by the lack of induction of the double-stranded RNA, interferon-inducible protein kinase, PKR.

In one embodiment, modifications are made to a double-stranded RNA molecule which would normally activate the interferon pathway such that the interferon pathway is not activated. For example, the interferon pathway is activated by double-stranded unmodified RNA. The cellular recognition of double-stranded RNA is highly specific and modifying one or both of the strands of a double-stranded duplex enable the double-stranded RNA molecule to evade the double-stranded RNA recognition machinery of the cell but would still allow for the activation of the RNAi pathway.

The ability of a double-stranded oligonucleotide to activate interferon could be assessed by testing for expression of the double-stranded RNA, Interferon-Inducible Protein Kinase, PKR using techniques known in the art and also testing for the ability of the double-stranded molecule to effect target gene inhibition. Accordingly, in one embodiment, the invention provides a method of testing for the ability of a double-stranded RNA molecule to induce interferon by testing for the ability of the oligonucleotide to activate PKR. Compositions that do not activate PKR (*i.e.*, do not activate the interferon pathway) are then selected for use to inhibit gene transcription in cells, *e.g.*, in therapeutics or functional genomics.

Without being limited to any particular mechanism of action, an antisense sequence used in a double-stranded oligonucleotide composition of the invention that can specifically hybridize with a nucleotide sequence within the target gene (*i.e.*, can be complementary to a nucleotide sequence within the target gene) may achieve its affects based on, *e.g.*: (1) binding to target mRNA and sterically blocking the ribosome complex from translating the mRNA; (2) binding to target mRNA and triggering mRNA cleavage by RNase H; (3) binding to double-stranded DNA in the nucleus and forming a triple helix; (4) hybridizing to open DNA loops created by RNA polymerase; (5) interfering with mRNA splicing; (6) interfering with transport of mRNA from the nucleus to the cytoplasm; or (7) interfering with translation through inhibition of the binding of initiation factors or assembly of ribosomal subunits (*i.e.*, at the start codon).

In one embodiment, an antisense sequence of the double-stranded oligonucleotides of the invention is complementary to a target nucleic acid sequence over at least about 80% of the length of the antisense sequence. In another embodiment, the antisense sequence of the double-stranded oligonucleotide of the invention is complementary to a target nucleic acid sequence over at least about 90-95 % of the length of the antisense sequence. In another embodiment, the antisense sequence of the double-stranded oligonucleotide of the invention is complementary to a target nucleic acid sequence over the entire length of the antisense sequence.

In yet another embodiment, an antisense sequence of the double-stranded oligonucleotide hybridizes to at least a portion of the target gene under stringent hybridization conditions.

In one embodiment, antisense sequences of the invention are substantially complementary to a target nucleic acid sequence. In one embodiment, an antisense RNA molecule comprises a nucleotide sequence which is at least about 100 % complementary to a portion of the target gene. In another embodiment, an antisense RNA molecule comprises a nucleotide sequence which is at least about 90 % complementary to a portion of the target gene. In another embodiment, an antisense RNA molecule comprises a nucleotide sequence which is at least about 80 % complementary to a portion of the target gene. In another embodiment, an antisense RNA molecule comprises a nucleotide sequence which is at least about 60 % complementary to a portion of the target gene. In another embodiment, an antisense RNA molecule comprises a nucleotide sequence which is at least about 100 % complementary to a portion of the target gene. Preferably, no loops greater than about 8 nucleotides are formed by areas of non-complementarity between the oligonucleotide and the target.

In one embodiment, an antisense nucleotide sequence of the invention is complementary to a target nucleic acid sequence over at least about 80% of the length of the antisense sequence. In another embodiment, an antisense sequence of the invention is complementary to a target nucleic acid sequence over at least about 90-95 % of the length of the antisense sequence. In another embodiment, an antisense sequence of the invention is complementary to a target nucleic acid sequence over the entire length of the antisense sequence.

The antisense sequences used in an oligonucleotide composition of the invention may be of any type, *e.g.*, including morpholino oligonucleotides, RNase H activating oligonucleotides, or ribozymes.

In one embodiment, a double-stranded oligonucleotide of the invention can comprise (*i.e.*, be a duplex of) one nucleic acid molecule which is DNA and one nucleic acid molecule which is RNA.

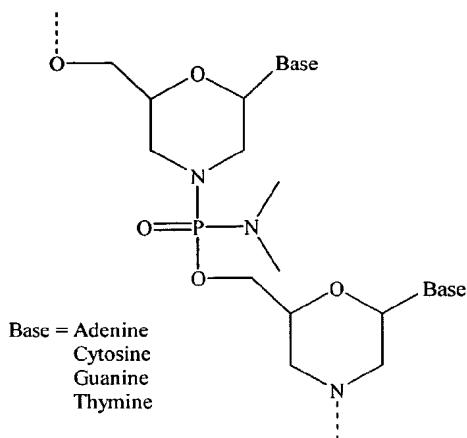
Antisense sequences of the invention can be “chimeric oligonucleotides” which comprise an RNA-like and a DNA-like region. The language “RNase H activating region” includes a region of an oligonucleotide, *e.g.*, a chimeric oligonucleotide, that is capable of recruiting RNase H to cleave the target RNA strand to which the oligonucleotide binds.

- 5      Typically, the RNase activating region contains a minimal core (of at least about 3-5, typically between about 3-12, more typically, between about 5-12, and more preferably between about 5-10 contiguous nucleomonomers) of DNA or DNA-like nucleomonomers. (*See, e.g.*, US patent 5,849,902). Preferably, the RNase H activating region comprises about nine contiguous deoxyribose containing nucleomonomers.

- 10     In one embodiment, the contiguous nucleomonomers are linked by a substitute linkage, *e.g.*, a phosphorothioate linkage. In one embodiment, an antisense sequence of the invention is unstable, *i.e.*, is degraded in a cell, in the absence of the second strand (or self complementary sequence) which forms a double-stranded oligonucleotide of the invention. For example, in one embodiment, a chimeric antisense sequence comprises unmodified DNA  
15    nucleomonomers in the gap rather than phosphorothioate DNA.

- The language “non-activating region” includes a region of an antisense sequence, *e.g.*, a chimeric oligonucleotide, that does not recruit or activate RNase H. Preferably, a non-activating region does not comprise phosphorothioate DNA. The oligonucleotides of the invention comprise at least one non-activating region. In one embodiment, the non-activating  
20    region can be stabilized against nucleases or can provide specificity for the target by being complementary to the target and forming hydrogen bonds with the target nucleic acid molecule, which is to be bound by the oligonucleotide.

- Antisense sequences of the present invention may include “morpholino  
25    oligonucleotides.” Morpholino oligonucleotides are non-ionic and function by an RNase H-independent mechanism. Each of the 4 genetic bases (Adenine, Cytosine, Guanine, and Thymine/Uracil) of the morpholino oligonucleotides is linked to a 6-membered morpholine ring. Morpholino oligonucleotides are made by joining the 4 different subunit types by, *e.g.*, non-ionic phosphorodiamidate inter-subunit linkages. An example of a 2 subunit morpholino oligonucleotide is shown below.

*Figure 13.*

5 Morpholino oligonucleotides have many advantages including: complete resistance to nucleases (Antisense & Nuc. Acid Drug Dev. 1996. 6:267); predictable targeting (Biochimica Biophysica Acta. 1999. 1489:141); reliable activity in cells (Antisense & Nuc. Acid Drug Dev. 1997. 7:63); excellent sequence specificity (Antisense & Nuc. Acid Drug Dev. 1997. 7:151); minimal non-antisense activity (Biochimica Biophysica Acta. 1999. 1489:141); and simple osmotic or scrape delivery (Antisense & Nuc. Acid Drug Dev. 1997. 7:291). Morpholino oligonucleotides are also preferred because of their non-toxicity at high doses. A discussion of the preparation of morpholino oligonucleotides can be found in Antisense & Nuc. Acid Drug Dev. 1997. 7:187.

10

15 *Uptake Of Oligonucleotides By Cells*

Oligonucleotides and oligonucleotide compositions are contacted with (*i.e.*, brought into contact with, also referred to herein as administered or delivered to) and taken up by one or more cells or a cell lysate. The term “cells” includes prokaryotic and eukaryotic cells, preferably vertebrate cells, and, more preferably, mammalian cells. In a preferred embodiment, the oligonucleotide compositions of the invention are contacted with human cells.

20

Oligonucleotide compositions of the invention can be contacted with cells *in vitro*, e.g., in a test tube or culture dish, (and may or may not be introduced into a subject) or *in vivo*, e.g., in a subject such as a mammalian subject. Oligonucleotides are taken up by cells at a slow rate by endocytosis, but endocytosed oligonucleotides are generally sequestered and not available, e.g., for hybridization to a target nucleic acid molecule. In one embodiment, cellular uptake can be facilitated by electroporation or calcium phosphate precipitation. However, these procedures are only useful for *in vitro* or *ex vivo* embodiments, are not convenient and, in some cases, are associated with cell toxicity.

In another embodiment, delivery of oligonucleotides into cells can be enhanced by suitable art recognized methods including calcium phosphate, DMSO, glycerol or dextran, electroporation, or by transfection, e.g., using cationic, anionic, or neutral lipid compositions or liposomes using methods known in the art (see e.g., WO 90/14074; WO 91/16024; WO 91/17424; U.S. Patent No. 4,897,355; Bergan *et al.* 1993. *Nucleic Acids Research*. 21:3567). Enhanced delivery of oligonucleotides can also be mediated by the use of vectors (See e.g., Shi, Y. 2003. Trends Genet 2003 Jan 19:9; Reichhart JM *et al.* Genesis. 2002. 34(1-2):160-4, Yu *et al.* 2002. Proc Natl Acad Sci U S A 99:6047; Sui *et al.* 2002. Proc Natl Acad Sci U S A 99:5515) viruses, polyamine or polycation conjugates using compounds such as polylysine, protamine, or N1, N12-bis (ethyl) spermine (see, e.g., Bartzatt, R. *et al.* 1989. *Biotechnol. Appl. Biochem.* 11:133; Wagner E. *et al.* 1992. *Proc. Natl. Acad. Sci.* 88:4255)

20

### Conjugating Agents

Conjugating agents bind to the oligonucleotide in a covalent manner. In one embodiment, oligonucleotides can be derivitized or chemically modified by binding to a conjugating agent to facilitate cellular uptake. For example, covalent linkage of a cholesterol moiety to an oligonucleotide can improve cellular uptake by 5- to 10- fold which in turn improves DNA binding by about 10- fold (Boutorin *et al.*, 1989, *FEBS Letters* 254:129-132). Conjugation of octyl, dodecyl, and octadecyl residues enhances cellular uptake by 3-, 4-, and 10- fold as compared to unmodified oligonucleotides (Vlassov *et al.*, 1994, *Biochimica et Biophysica Acta* 1197:95-108). Similarly, derivatization of oligonucleotides with poly-L-lysine can aid oligonucleotide uptake by cells (Schell, 1974, *Biochem. Biophys. Acta* 340:323, and Lemaitre *et al.*, 1987, *Proc. Natl. Acad. Sci. USA* 84:648).

Certain protein carriers can also facilitate cellular uptake of oligonucleotides, including, for example, serum albumin, nuclear proteins possessing signals for transport to the nucleus, and viral or bacterial proteins capable of cell membrane penetration. Therefore, protein carriers are useful when associated with or linked to the oligonucleotides.

- 5 Accordingly, the present invention provides for derivatization of oligonucleotides with groups capable of facilitating cellular uptake, including hydrocarbons and non-polar groups, cholesterol, long chain alcohols (*i.e.*, hexanol), poly-L-lysine and proteins, as well as other aryl or steroid groups and polycations having analogous beneficial effects, such as phenyl or naphthyl groups, quinoline, anthracene or phenanthracene groups, fatty acids, fatty alcohols 10 and sesquiterpenes, diterpenes, and steroids. A major advantage of using conjugating agents is to increase the initial membrane interaction that leads to a greater cellular accumulation of oligonucleotides.

#### Encapsulating Agents

- 15 Encapsulating agents entrap oligonucleotides within vesicles. In another embodiment of the invention, an oligonucleotide may be associated with a carrier or vehicle, *e.g.*, liposomes or micelles, although other carriers could be used, as would be appreciated by one skilled in the art. Liposomes are vesicles made of a lipid bilayer having a structure similar to biological membranes. Such carriers are used to facilitate the cellular uptake or targeting of 20 the oligonucleotide, or improve the oligonucleotide's pharmacokinetic or toxicologic properties.

- For example, the oligonucleotides of the present invention may also be administered encapsulated in liposomes, pharmaceutical compositions wherein the active ingredient is contained either dispersed or variously present in corpuscles consisting of aqueous concentric 25 layers adherent to lipidic layers. The oligonucleotides, depending upon solubility, may be present both in the aqueous layer and in the lipidic layer, or in what is generally termed a liposomal suspension. The hydrophobic layer, generally but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, more or less 30 ionic surfactants such as diacetylphosphate, stearylamine, or phosphatidic acid, or other materials of a hydrophobic nature. The diameters of the liposomes generally range from about 15 nm to about 5 microns.

The use of liposomes as drug delivery vehicles offers several advantages. Liposomes increase intracellular stability, increase uptake efficiency and improve biological activity. Liposomes are hollow spherical vesicles composed of lipids arranged in a similar fashion as those lipids which make up the cell membrane. They have an internal aqueous space for entrapping water soluble compounds and range in size from 0.05 to several microns in diameter. Several studies have shown that liposomes can deliver nucleic acids to cells and that the nucleic acids remain biologically active. For example, a liposome delivery vehicle originally designed as a research tool, such as Lipofectin, can deliver intact nucleic acid molecules to cells.

Specific advantages of using liposomes include the following: they are non-toxic and biodegradable in composition; they display long circulation half-lives; and recognition molecules can be readily attached to their surface for targeting to tissues. Finally, cost-effective manufacture of liposome-based pharmaceuticals, either in a liquid suspension or lyophilized product, has demonstrated the viability of this technology as an acceptable drug delivery system.

#### Complexing Agents

Complexing agents bind to the oligonucleotides of the invention by a strong but non-covalent attraction (e.g., an electrostatic, van der Waals, pi-stacking, etc. interaction). In one embodiment, oligonucleotides of the invention can be complexed with a complexing agent to increase cellular uptake of oligonucleotides. An example of a complexing agent includes cationic lipids. Cationic lipids can be used to deliver oligonucleotides to cells.

The term "cationic lipid" includes lipids and synthetic lipids having both polar and non-polar domains and which are capable of being positively charged at or around physiological pH and which bind to polyanions, such as nucleic acids, and facilitate the delivery of nucleic acids into cells. In general cationic lipids include saturated and unsaturated alkyl and alicyclic ethers and esters of amines, amides, or derivatives thereof. Straight-chain and branched alkyl and alkenyl groups of cationic lipids can contain, e.g., from 1 to about 25 carbon atoms. Preferred straight chain or branched alkyl or alkene groups have six or more carbon atoms. Alicyclic groups include cholesterol and other steroid groups. Cationic lipids can be prepared with a variety of counterions (anions) including, e.g., Cl<sup>-</sup>, Br<sup>-</sup>, I<sup>-</sup>, F<sup>-</sup>, acetate, trifluoroacetate, sulfate, nitrite, and nitrate.

Examples of cationic lipids include polyethylenimine, polyamidoamine (PAMAM) starburst dendrimers, Lipofectin (a combination of DOTMA and DOPE), Lipofectase, Lipofectamine, DOPE, Cytofectin (Gilead Sciences, Foster City, CA), and Eufectins (JBL, San Luis Obispo, CA). Exemplary cationic liposomes can be made from N-[1-(2,3-dioleolxy)-propyl]-N,N,N-trimethylammonium chloride (DOTMA), N-[1-(2,3-dioleolxy)-propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP), 3 $\beta$ -[N-(N',N'-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol), 2,3,-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide; and dimethyldioctadecylammonium bromide (DDAB). The cationic lipid N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), for example, was found to increase 1000-fold the antisense effect of a phosphorothioate oligonucleotide. (Vlassov *et al.*, 1994, *Biochimica et Biophysica Acta* 1197:95-108). Oligonucleotides can also be complexed with, *e.g.*, poly (L-lysine) or avidin and lipids may, or may not, be included in this mixture, *e.g.*, steryl-poly (L-lysine).

Cationic lipids have been used in the art to deliver oligonucleotides to cells (*see, e.g.*, U.S. 5,855,910; 5,851,548; 5,830,430; 5,780,053; 5,767,099; Lewis *et al.* 1996. *Proc. Natl. Acad. Sci. USA* 93:3176; Hope *et al.* 1998. *Molecular Membrane Biology* 15:1). Other lipid compositions which can be used to facilitate uptake of the instant oligonucleotides can be used in connection with the claimed methods. In addition to those listed supra, other lipid compositions are also known in the art and include, *e.g.*, those taught in U.S. patent 4,235,871; U.S. patents 4,501,728; 4,837,028; 4,737,323.

In one embodiment lipid compositions can further comprise agents, *e.g.*, viral proteins to enhance lipid-mediated transfections of oligonucleotides (Kamata, *et al.*, 1994. *Nucl. Acids. Res.* 22:536). In another embodiment, oligonucleotides are contacted with cells as part of a composition comprising an oligonucleotide, a peptide, and a lipid as taught, *e.g.*, in U.S. patent 5,736,392. Improved lipids have also been described which are serum resistant (Lewis, *et al.*, 1996. *Proc. Natl. Acad. Sci.* 93:3176). Cationic lipids and other complexing agents act to increase the number of oligonucleotides carried into the cell through endocytosis.

In another embodiment N-substituted glycine oligonucleotides (peptoids) can be used to optimize uptake of oligonucleotides. Peptoids have been used to create cationic lipid-like compounds for transfection (Murphy, *et al.*, 1998. *Proc. Natl. Acad. Sci.* 95:1517). Peptoids can be synthesized using standard methods (*e.g.*, Zuckermann, R. N., *et al.* 1992. *J. Am. Chem. Soc.* 114:10646; Zuckermann, R.N., *et al.* 1992. *Int. J. Peptide Protein Res.* 40:497). Combinations of cationic lipids and peptoids, liptoids, can also be used to optimize uptake of the subject oligonucleotides (Hunag ,*et al.*, 1998. *Chemistry and Biology.* 5:345). Liptoids can be synthesized by elaborating peptoid oligonucleotides and coupling the amino terminal submonomer to a lipid via its amino group (Hunag, *et al.*, 1998. *Chemistry and Biology.* 5:345).

It is known in the art that positively charged amino acids can be used for creating highly active cation lipids (Lewis *et al.* 1996. *Proc. Natl. Acad. Sci. U.S.A.* 93:3176). In one embodiment, a composition for delivering oligonucleotides of the invention comprises a number of arginine, lysine, histidine or ornithine residues linked to a lipophilic moiety (see *e.g.*, U.S. patent 5,777,153).

In another, a composition for delivering oligonucleotides of the invention comprises a peptide having from between about one to about four basic residues. These basic residues can be located, *e.g.*, on the amino terminal, C-terminal, or internal region of the peptide. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine (can also be considered non-polar), asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Apart from the basic amino acids, a majority or all of the other residues of the peptide can be selected from the non-basic amino acids, *e.g.*, amino acids other than lysine, arginine, or histidine. Preferably a preponderance of neutral amino acids with long neutral side chains are used. For example, a peptide such as (N-term) His-Ile-Trp-Leu-Ile-Tyr-Leu-Trp-Ile-Val-(C-term) (SEQ ID NO: ##) could be used. In one embodiment such a composition can be mixed with the fusogenic lipid DOPE as is well known in the art.

In one embodiment, the cells to be contacted with an oligonucleotide composition of the invention are contacted with a mixture comprising the oligonucleotide and a mixture comprising a lipid, e.g., one of the lipids or lipid compositions described supra for between about 12 h to about 24 h. In another embodiment, the cells to be contacted with an  
5 oligonucleotide composition are contacted with a mixture comprising the oligonucleotide and a mixture comprising a lipid, e.g., one of the lipids or lipid compositions described supra for between about 1 and about five days. In one embodiment, the cells are contacted with a mixture comprising a lipid and the oligonucleotide for between about three days to as long as about 30 days. In another embodiment, a mixture comprising a lipid is left in contact with the  
10 cells for at least about five to about 20 days. In another embodiment, a mixture comprising a lipid is left in contact with the cells for at least about seven to about 15 days.

For example, in one embodiment, an oligonucleotide composition can be contacted with cells in the presence of a lipid such as cytofectin CS or GSV(available from Glen Research; Sterling, VA), GS3815, GS2888 for prolonged incubation periods as described  
15 herein.

In one embodiment the incubation of the cells with the mixture comprising a lipid and an oligonucleotide composition does not reduce the viability of the cells. Preferably, after the transfection period the cells are substantially viable. In one embodiment, after transfection, the cells are between at least about 70 and at least about 100 percent viable. In another  
20 embodiment, the cells are between at least about 80 and at least about 95% viable. In yet another embodiment, the cells are between at least about 85% and at least about 90% viable.

In one embodiment, oligonucleotides are modified by attaching a peptide sequence that transports the oligonucleotide into a cell, referred to herein as a “transporting peptide.” In one embodiment, the composition includes an oligonucleotide which is complementary to  
25 a target nucleic acid molecule encoding the protein, and a covalently attached transporting peptide.

The language “transporting peptide” includes an amino acid sequence that facilitates the transport of an oligonucleotide into a cell. Exemplary peptides which facilitate the transport of the moieties to which they are linked into cells are known in the art, and include,  
30 e.g., HIV TAT transcription factor, lactoferrin, Herpes VP22 protein, and fibroblast growth factor 2 (Pooga *et al.* 1998. *Nature Biotechnology*. 16:857; and Derossi *et al.* 1998. *Trends in Cell Biology*. 8:84; Elliott and O’Hare. 1997. *Cell* 88:223).

For example, in one embodiment, the transporting peptide comprises an amino acid sequence derived from the antennapedia protein. Preferably, the peptide comprises amino acids 43-58 of the antennapedia protein (Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys) (SEQ ID NO: ##) or a portion or variant thereof that facilitates transport of an oligonucleotide into a cell (see, e.g., WO 91/1898; Derossi *et al.* 1998. *Trends Cell Biol.* 8:84). Exemplary variants are shown in Derossi *et al.*, supra.

In one embodiment, the transporting peptide comprises an amino acid sequence derived from the transportan, galanin (1-12)-Lys-mastoparan (1-14) amide, protein. (Pooga *et al.* 1998. *Nature Biotechnology* 16:857). Preferably, the peptide comprises the amino acids of the transportan protein shown in the sequence GWTLNSAGYLLGKINLKAL-AALAKKIL (SEQ ID NO: ##) or a portion or variant thereof that facilitates transport of an oligonucleotide into a cell.

In one embodiment, the transporting peptide comprises an amino acid sequence derived from the HIV TAT protein. Preferably, the peptide comprises amino acids 37-72 of the HIV TAT protein, e.g., shown in the sequence C(Acm)FITKALGISYGRKKRRQRRR-PPQC (SEQ ID NO: ##) (TAT 37-60; where C(Acm) is Cys-acetamidomethyl) or a portion or variant thereof, e.g., C(Acm)GRKKRRQRRRPPQC (SEQ ID NO: ##) (TAT 48-40) or C(Acm)LGISYGRKKRRQRRPPQC (SEQ ID NO: ##) (TAT 43-60) that facilitates transport of an oligonucleotide into a cell (Vives *et al.* 1997. *J. Biol. Chem.* 272:16010). In another embodiment the peptide (G)CFITKALGISYGRKKRRQR-RRPPQGSQTHQVSLSKQ (SEQ ID NO: ##) can be used.

Portions or variants of transporting peptides can be readily tested to determine whether they are equivalent to these peptide portions by comparing their activity to the activity of the native peptide, e.g., their ability to transport fluorescently-labeled oligonucleotides to cells. Fragments or variants that retain the ability of the native transporting peptide to transport an oligonucleotide into a cell are functionally equivalent and can be substituted for the native peptides.

Oligonucleotides can be attached to the transporting peptide using known techniques, e.g., ( Prochiantz, A. 1996. *Curr. Opin. Neurobiol.* 6:629; Derossi *et al.* 1998. *Trends Cell Biol.* 8:84; Troy *et al.* 1996. *J. Neurosci.* 16:253), Vives *et al.* 1997. *J. Biol. Chem.* 272:16010). For example, in one embodiment, oligonucleotides bearing an activated thiol group are linked via that thiol group to a cysteine present in a transport peptide (e.g., to the cysteine present in the  $\beta$  turn between the second and the third helix of the antennapedia homeodomain as taught, e.g., in Derossi *et al.* 1998. *Trends Cell Biol.* 8:84; Prochiantz. 1996. *Current Opinion in Neurobiol.* 6:629; Allinquant *et al.* 1995. *J. Cell Biol.* 128:919). In another embodiment, a Boc-Cys-(Npys)OH group can be coupled to the transport peptide as the last (N-terminal) amino acid and an oligonucleotide bearing an SH group can be coupled to the peptide (Troy *et al.* 1996. *J. Neurosci.* 16:253).

In one embodiment, a linking group can be attached to a nucleomonomer and the transporting peptide can be covalently attached to the linker. In one embodiment, a linker can function as both an attachment site for a transporting peptide and can provide stability against nucleases. Examples of suitable linkers include substituted or unsubstituted C<sub>1</sub>-C<sub>20</sub> alkyl chains, C<sub>2</sub>-C<sub>20</sub> alkenyl chains, C<sub>2</sub>-C<sub>20</sub> alkynyl chains, peptides, and heteroatoms (e.g., S, O, NH, etc.). Other exemplary linkers include bifunctional crosslinking agents such as sulfosuccinimidyl-4-(maleimidophenyl)-butyrate (SMPB) (see, e.g., Smith *et al.* Biochem J 1991. 276: 417-2).

In one embodiment, oligonucleotides of the invention are synthesized as molecular conjugates which utilize receptor-mediated endocytotic mechanisms for delivering genes into cells (see, e.g., Bunnell *et al.* 1992. *Somatic Cell and Molecular Genetics.* 18:559 and the references cited therein).

25     *Targeting Agents*

The delivery of oligonucleotides can also be improved by targeting the oligonucleotides to a cellular receptor. The targeting moieties can be conjugated to the oligonucleotides or attached to a carrier group (*i.e.*, poly(L-lysine) or liposomes) linked to the oligonucleotides. This method is well suited to cells that display specific receptor-mediated 30 endocytosis.

For instance, oligonucleotide conjugates to 6-phosphomannosylated proteins are internalized 20-fold more efficiently by cells expressing mannose 6-phosphate specific receptors than free oligonucleotides. The oligonucleotides may also be coupled to a ligand for a cellular receptor using a biodegradable linker. In another example, the delivery 5 construct is mannosylated streptavidin which forms a tight complex with biotinylated oligonucleotides. Mannosylated streptavidin was found to increase 20-fold the internalization of biotinylated oligonucleotides. (Vlassov *et al.* 1994. *Biochimica et Biophysica Acta* 1197:95-108).

In addition specific ligands can be conjugated to the polylysine component of 10 polylysine-based delivery systems. For example, transferrin-polylysine, adenovirus-polylysine, and influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides-polylysine conjugates greatly enhance receptor-mediated DNA delivery in eucaryotic cells. Mannosylated glycoprotein conjugated to poly(L-lysine) in aveolar macrophages has been employed to enhance the cellular uptake of oligonucleotides. Liang *et al.* 1999. *Pharmazie* 15 54:559-566.

Because malignant cells have an increased need for essential nutrients such as folic acid and transferrin, these nutrients can be used to target oligonucleotides to cancerous cells. For example, when folic acid is linked to poly(L-lysine) enhanced oligonucleotide uptake is seen in promyelocytic leukaemia (HL-60) cells and human melanoma (M-14) cells. Ginobbi 20 *et al.* 1997. *Anticancer Res.* 17:29. In another example, liposomes coated with maleylated bovine serum albumin, folic acid, or ferric protoporphyrin IX, show enhanced cellular uptake of oligonucleotides in murine macrophages, KB cells, and 2.2.15 human hepatoma cells. Liang *et al.* 1999. *Pharmazie* 54:559-566.

Liposomes naturally accumulate in the liver, spleen, and reticuloendothelial system 25 (so-called, passive targeting). By coupling liposomes to various ligands such as antibodies are protein A, they can be actively targeted to specific cell populations. For example, protein A-bearing liposomes may be pretreated with H-2K specific antibodies which are targeted to the mouse major histocompatibility complex-encoded H-2K protein expressed on L cells. (Vlassov *et al.* 1994. *Biochimica et Biophysica Acta* 1197:95-108).

Assays of Oligonucleotide Stability

Preferably, the double-stranded oligonucleotides of the invention are stabilized, *i.e.*, substantially resistant to endonuclease and exonuclease degradation. An oligonucleotide is defined as being substantially resistant to nucleases when it is at least about 3-fold more  
5 resistant to attack by an endogenous cellular nuclease, and is highly nuclease resistant when it is at least about 6-fold more resistant than a corresponding, single-stranded oligonucleotide. This can be demonstrated by showing that the oligonucleotides of the invention are substantially resist nucleases using techniques which are known in the art.

One way in which substantial stability can be demonstrated is by showing that the  
10 oligonucleotides of the invention function when delivered to a cell, *e.g.*, that they reduce transcription or translation of target nucleic acid molecules, *e.g.*, by measuring protein levels or by measuring cleavage of mRNA. Assays which measure the stability of target RNA can be performed at about 24 hours post-transfection (*e.g.*, using Northern blot techniques, RNase Protection Assays, or QC-PCR assays as known in the art). Alternatively, levels of the target  
15 protein can be measured. Preferably, in addition to testing the RNA or protein levels of interest, the RNA or protein levels of a control, non-targeted gene will be measured (*e.g.*, actin, or preferably a control with sequence similarity to the target) as a specificity control. RNA or protein measurements can be made using any art-recognized technique. Preferably, measurements will be made beginning at about 16-24 hours post transfection. (M. Y. Chiang,  
20 *et al.* 1991. *J Biol Chem.* 266:18162-71; T. Fisher, *et al.* 1993. *Nucleic Acids Research.* 21 3857).

The ability of an oligonucleotide composition of the invention to inhibit protein synthesis can be measured using techniques which are known in the art, for example, by detecting an inhibition in gene transcription or protein synthesis. For example, Nuclease S1  
25 mapping can be performed. In another example, Northern blot analysis can be used to measure the presence of RNA encoding a particular protein. For example, total RNA can be prepared over a cesium chloride cushion (*see, e.g.*, Ausubel *et al.*, 1987. Current Protocols in Molecular Biology (Greene & Wiley, New York)). Northern blots can then be made using the RNA and probed (*see, e.g.*, Id.). In another example, the level of the specific mRNA  
30 produced by the target protein can be measured, *e.g.*, using PCR. In yet another example, Western blots can be used to measure the amount of target protein present. In still another embodiment, a phenotype influenced by the amount of the protein can be detected. Techniques for performing Western blots are well known in the art, *see, e.g.*, Chen *et al.* *J. Biol. Chem.* 271:28259.

In another example, the promoter sequence of a target gene can be linked to a reporter gene and reporter gene transcription (*e.g.*, as described in more detail below) can be monitored. Alternatively, oligonucleotide compositions that do not target a promoter can be identified by fusing a portion of the target nucleic acid molecule with a reporter gene so that 5 the reporter gene is transcribed. By monitoring a change in the expression of the reporter gene in the presence of the oligonucleotide composition, it is possible to determine the effectiveness of the oligonucleotide composition in inhibiting the expression of the reporter gene. For example, in one embodiment, an effective oligonucleotide composition will reduce the expression of the reporter gene.

10 A “reporter gene” is a nucleic acid that expresses a detectable gene product, which may be RNA or protein. Detection of mRNA expression may be accomplished by Northern blotting and detection of protein may be accomplished by staining with antibodies specific to the protein. Preferred reporter genes produce a readily detectable product. A reporter gene may be operably linked with a regulatory DNA sequence such that detection of the reporter 15 gene product provides a measure of the transcriptional activity of the regulatory sequence. In preferred embodiments, the gene product of the reporter gene is detected by an intrinsic activity associated with that product. For instance, the reporter gene may encode a gene product that, by enzymatic activity, gives rise to a detectable signal based on color, fluorescence, or luminescence. Examples of reporter genes include, but are not limited to, 20 those coding for chloramphenicol acetyl transferase (CAT), luciferase,  $\beta$ -galactosidase, and alkaline phosphatase.

One skilled in the art would readily recognize numerous reporter genes suitable for use in the present invention. These include, but are not limited to, chloramphenicol acetyltransferase (CAT), luciferase, human growth hormone (hGH), and beta-galactosidase. 25 Examples of such reporter genes can be found in F. A. Ausubel *et al.*, Eds., Current Protocols in Molecular Biology, John Wiley & Sons, New York, (1989). Any gene that encodes a detectable product, *e.g.*, any product having detectable enzymatic activity or against which a specific antibody can be raised, can be used as a reporter gene in the present methods.

One reporter gene system is the firefly luciferase reporter system. (Gould, S. J., and 30 Subramani, S. 1988. Anal. Biochem., 7:404-408 incorporated herein by reference). The luciferase assay is fast and sensitive. In this assay, a lysate of the test cell is prepared and combined with ATP and the substrate luciferin. The encoded enzyme luciferase catalyzes a rapid, ATP dependent oxidation of the substrate to generate a light-emitting product. The total light output is measured and is proportional to the amount of luciferase present over a 35 wide range of enzyme concentrations.

CAT is another frequently used reporter gene system; a major advantage of this system is that it has been an extensively validated and is widely accepted as a measure of promoter activity. (Gorman C. M., Moffat, L. F., and Howard, B. H. 1982. Mol. Cell. Biol., 2:1044-1051). In this system, test cells are transfected with CAT expression vectors and 5 incubated with the candidate substance within 2-3 days of the initial transfection. Thereafter, cell extracts are prepared. The extracts are incubated with acetyl CoA and radioactive chloramphenicol. Following the incubation, acetylated chloramphenicol is separated from nonacetylated form by thin layer chromatography. In this assay, the degree of acetylation reflects the CAT gene activity with the particular promoter.

10 Another suitable reporter gene system is based on immunologic detection of hGH. This system is also quick and easy to use. (Selden, R., Burke-Howie, K. Rowe, M. E., Goodman, H. M., and Moore, D. D. (1986), Mol. Cell, Biol., 6:3173-3179 incorporated herein by reference). The hGH system is advantageous in that the expressed hGH polypeptide is assayed in the media, rather than in a cell extract. Thus, this system does not 15 require the destruction of the test cells. It will be appreciated that the principle of this reporter gene system is not limited to hGH but rather adapted for use with any polypeptide for which an antibody of acceptable specificity is available or can be prepared.

20 In one embodiment, nuclease stability of a double-stranded oligonucleotide of the invention is measured and compared to a control, e.g., an RNAi molecule typically used in the art (e.g., a duplex oligonucleotide of less than 25 nucleotides in length and comprising 2 nucleotide base overhangs) or an unmodified RNA duplex with blunt ends.

### Oligonucleotide Synthesis

25 Oligonucleotides of the invention can be synthesized by any method known in the art, e.g., using enzymatic synthesis and chemical synthesis. The oligonucleotides can be synthesized *in vitro* (e.g., using enzymatic synthesis and chemical synthesis) or *in vivo* (using recombinant DNA technology well known in the art).

30 In a preferred embodiment, chemical synthesis is used. Chemical synthesis of linear oligonucleotides is well known in the art and can be achieved by solution or solid phase techniques. Preferably, synthesis is by solid phase methods. Oligonucleotides can be made by any of several different synthetic procedures including the phosphoramidite, phosphite triester, H-phosphonate, and phosphotriester methods, typically by automated synthesis methods.

Oligonucleotide synthesis protocols are well known in the art and can be found, e.g., in U.S. patent 5,830,653; WO 98/13526; Stec *et al.* 1984. *J. Am. Chem. Soc.* 106:6077; Stec *et al.* 1985. *J. Org. Chem.* 50:3908; Stec *et al.* *J. Chromatog.* 1985. 326:263; LaPlanche *et al.* 1986. *Nuc. Acid. Res.* 1986. 14:9081; Fasman G. D., 1989. *Practical Handbook of Biochemistry and Molecular Biology.* 1989. CRC Press, Boca Raton, Fla.; Lamone. 1993. *Biochem. Soc. Trans.* 21:1; U.S. Patent 5,013,830; U.S. Patent 5,214,135; U.S. Patent 5,525,719; Kawasaki *et al.* 1993. *J. Med. Chem.* 36:831; WO 92/03568; U.S. Patent 5,276,019; U.S. Patent 5,264,423.

The synthesis method selected can depend on the length of the desired oligonucleotide and such choice is within the skill of the ordinary artisan. For example, the phosphoramidite and phosphite triester method can produce oligonucleotides having 175 or more nucleotides while the H-phosphonate method works well for oligonucleotides of less than 100 nucleotides. If modified bases are incorporated into the oligonucleotide, and particularly if modified phosphodiester linkages are used, then the synthetic procedures are altered as needed according to known procedures. In this regard, Uhlmann *et al.* (1990, *Chemical Reviews* 90:543-584) provide references and outline procedures for making oligonucleotides with modified bases and modified phosphodiester linkages. Other exemplary methods for making oligonucleotides are taught in Sonveaux. 1994. "Protecting Groups in Oligonucleotide Synthesis"; Agrawal. *Methods in Molecular Biology* 26:1. Exemplary synthesis methods are also taught in "Oligonucleotide Synthesis- A Practical Approach" (Gait, M.J. IRL Press at Oxford University Press. 1984). Moreover, linear oligonucleotides of defined sequence, including some sequences with modified nucleotides, are readily available from several commercial sources.

The oligonucleotides may be purified by polyacrylamide gel electrophoresis, or by any of a number of chromatographic methods, including gel chromatography and high pressure liquid chromatography. To confirm a nucleotide sequence, oligonucleotides may be subjected to DNA sequencing by any of the known procedures, including Maxam and Gilbert sequencing, Sanger sequencing, capillary electrophoresis sequencing the wandering spot sequencing procedure or by using selective chemical degradation of oligonucleotides bound to Hybond paper. Sequences of short oligonucleotides can also be analyzed by laser desorption mass spectroscopy or by fast atom bombardment (McNeal, *et al.*, 1982, *J. Am. Chem. Soc.* 104:976; Viari, *et al.*, 1987, *Biomed. Environ. Mass Spectrom.* 14:83; Grotjahn *et al.*, 1982, *Nuc. Acid Res.* 10:4671). Sequencing methods are also available for RNA oligonucleotides.

The quality of oligonucleotides synthesized can be verified by testing the oligonucleotide by capillary electrophoresis and denaturing strong anion HPLC (SAX-HPLC) using, e.g., the method of Bergot and Egan. 1992. *J. Chrom.* 599:35.

Other exemplary synthesis techniques are well known in the art (see, e.g., Sambrook *et al.*, Molecular Cloning: a Laboratory Manual, Second Edition (1989); DNA Cloning, Volumes I and II (DN Glover Ed. 1985); Oligonucleotide Synthesis (MJ Gait Ed, 1984; Nucleic Acid Hybridisation (BD Hames and SJ Higgins eds. 1984); A Practical Guide to Molecular Cloning (1984); or the series, Methods in Enzymology (Academic Press, Inc.)).

### Uses of Oligonucleotides

This invention also features methods of inhibiting expression of a protein in a cell including contacting the cell with one of the above-described oligonucleotide compositions.

The oligonucleotides of the invention can be used in a variety of *in vitro* and *in vivo* situations to specifically inhibit protein expression. The instant methods and compositions are suitable for both *in vitro* and *in vivo* use.

The methods of the invention may be used for determining the function of a gene in a cell or an organism or for modulating the function of a gene in a cell or an organism, being capable of responding to or mediating RNA interference. The cell is preferably a eukaryotic cell or a cell line, e.g., an animal cell such as a mammalian cell, e.g., an embryonic cell, a pluripotent stem cell, a tumor cell, e.g., a teratocarcinoma cell, or a virus-infected cell. The organism is preferably a eukaryotic organism, e.g., an animal such as a mammal, particularly a human.

The invention includes methods to inhibit expression of a target gene in a cell *in vitro*. For example, such methods may include introduction of RNA into a cell in an amount sufficient to inhibit expression of the target gene, where the RNA is a double-stranded molecule of the invention. By way of a further example, such an RNA molecule may have a first strand consisting essentially of a ribonucleotide sequence that corresponds to a nucleotide sequence of the target gene, and a second strand consisting essentially of a ribonucleotide sequence that is complementary to the nucleotide sequence of the target gene, in which the first and the second strands are separate complementary strands or are joined by a loop, and they hybridize to each other to form said double-stranded molecule, such that the duplex composition inhibits expression of the target gene. The duplex composition may include modified nucleomonomers as discussed above.

The invention also relates to a method to inhibit expression of a target gene in an invertebrate organism. Such methods include providing an invertebrate organism containing a target cell that contains the target gene, in which the target cell is susceptible to RNA interference and the target gene is expressed in the target cell. Such methods further include

5 contacting the invertebrate organism with an RNA composition of the invention. For example, the RNA may be a double-stranded molecule with a first strand consisting essentially of a ribonucleotide sequence that corresponds to a nucleotide sequence of the target gene and a second strand consisting essentially of a ribonucleotide sequence that is complementary to the nucleotide sequence of the target gene. In such cases, the first and the

10 second ribonucleotide sequences may be separate complementary strands or joined by a loop, and they hybridize to each other to form the double-stranded molecule. Finally, such methods include a step of introducing the duplex RNA composition into the target cell to thereby inhibiting expression of the target gene.

In one embodiment, the oligonucleotides of the invention can be used to inhibit gene function *in vitro* in a method for identifying the functions of genes. In this manner, the transcription of genes that are identified, but for which no function has yet been shown, can be inhibited to thereby determine how the phenotype of a cell is changed when the gene is not transcribed. Such methods are useful for the validation of genes as targets for clinical treatment, *e.g.*, with oligonucleotides or with other therapies.

To determine the effect of a composition of the invention, a variety of end points can be used. In addition to the assays described previously herein, for example, nucleic acid probes (*e.g.*, in the form of arrays) can be used to evaluate transcription patterns produced by cells. Probes can also be used detect peptides, proteins, or protein domains, *e.g.*, antibodies can be used to detect the expression of a particular protein. In yet another embodiment, the function of a protein (*e.g.*, enzymatic activity) can be measured. In yet another embodiment, the phenotype of a cell can be evaluated to determine whether or not a target protein is expressed. For example, the ability of a composition to affect a phenotype of a cell that is associated with cancer can be tested.

In one embodiment, one or more additional agents (*e.g.*, activating agents, inducing agents, proliferation enhancing agents, tumor promoters) can be added to the cells.

In another embodiment, the compositions of the invention can be used to monitor biochemical reactions such as, *e.g.*, interactions of proteins, nucleic acids, small molecules, or the like, for example the efficiency or specificity of interactions between antigens and antibodies; or of receptors (such as purified receptors or receptors bound to cell membranes) and their ligands, agonists or antagonists; or of enzymes (such as proteases or kinases) and their substrates, or increases or decreases in the amount of substrate converted to a product; as well as many others. Such biochemical assays can be used to characterize properties of the probe or target, or as the basis of a screening assay. For example, to screen samples for the presence of particular proteases (*e.g.*, proteases involved in blood clotting such as proteases Xa and VIIa), the samples can be assayed, for example using probes which are fluorogenic substrates specific for each protease of interest. If a target protease binds to and cleaves a substrate, the substrate will fluoresce, usually as a result, *e.g.*, of cleavage and separation between two energy transfer pairs, and the signal can be detected. In another example, to screen samples for the presence of a particular kinase(s) (*e.g.*, a tyrosine kinase), samples containing one or more kinases of interest can be assayed, *e.g.*, using probes are peptides which can be selectively phosphorylated by one of the kinases of interest. Using art-recognized, routinely determinable conditions, samples can be incubated with an array of substrates, in an appropriate buffer and with the necessary cofactors, for an empirically determined period of time. If necessary, reactions can be stopped, *e.g.*, by washing and the phosphorylated substrates can be detected by, for example, incubating them with detectable reagents such as, *e.g.*, fluorescein-labeled anti-phosphotyrosine or anti-phosphoserine antibodies and the signal can be detected.

In another embodiment, the compositions of the invention can be used to screen for agents which modulate a pattern of gene expression. Arrays of oligonucleotides can be used, for example, to identify mRNA species whose pattern of expression from a set of genes is correlated with a particular physiological state or developmental stage, or with a disease condition (“correlative” genes, RNAs, or expression patterns). By the terms “correlate” or “correlative,” it is meant that the synthesis pattern of RNA is associated with the physiological condition of a cell, but not necessarily that the expression of a given RNA is responsible for or is causative of a particular physiological state. For example, a small subset of mRNAs can be identified which are modulated (*e.g.*, upregulated or downregulated) in cells which serve as a model for a particular disease state. This altered pattern of expression as compared to that in a normal cell, which does not exhibit a pathological phenotype, can serve as a indicator of the disease state (“indicator” or “correlatvie” genes, RNAs, or expression patterns).

Compositions which modulate the chosen indicator expression pattern (*e.g.*, compared to control compositions comprising, for example oligonucleotides which comprise a nucleotide sequence which is the reverse of the oligonucleotide, or which contains mismatch bases) can indicate that a particular target gene is a potential target for therapeutic intervention. Moreover, such compositions may be useful as therapeutic agents to modulate expression patterns of cells in an *in vitro* expression system or in *in vivo* therapy. As used herein, “modulate” means to cause to increase or decrease the amount or activity of a molecule or the like which is involved in a measurable reaction. In one embodiment, a series of cells (*e.g.*, from a disease model) can be contacted with a series of agents (*e.g.*, for a period of time ranging from about 10 minutes to about 48 hours or more) and, using routine, art-recognized methods (*e.g.*, commercially available kits), total RNA or mRNA extracts can be made. If it is desired to amplify the amount of RNA, standard procedures such as RT-PCR amplification can be used (*see, e.g.*, Innis et al eds., (1996) PCR Protocols: A Guide to Methods in Amplification, Academic Press, New York). The extracts (or amplified products from them) can be allowed to contact (*e.g.*, incubate with) probes for appropriate indicator RNAs, and those agents which are associated with a change in the indicator expression pattern can be identified.

Similarly, agents can be identified which modulate expression patterns associated with particular physiological states or developmental stages. Such agents can be man-made or naturally-occurring substances, including environmental factors such as substances involved in embryonic development or in regulating physiological reactions.

In one embodiment, the methods described herein can be performed in a “high throughput” manner, in which a large number of target genes (*e.g.*, as many as about 1000 or more, depending on the particular format used) are assayed rapidly and concurrently. Further, many assay formats (*e.g.*, plates or surfaces) can be processed at one time. For example, because the oligonucleotides of the invention do not need to be tested individually before incorporating them into a composition, they can be readily synthesized and large numbers of target genes can be tested at one time. For example, a large number of samples, each comprising a biological sample containing a target nucleic acid molecule (*e.g.*, a cell) and a composition of the invention can be added to separate regions of an assay format and assays can be performed on each of the samples.

Administration of Oligonucleotide Compositions

The optimal course of administration or delivery of the oligonucleotides may vary depending upon the desired result and/ or on the subject to be treated. As used herein "administration" refers to contacting cells with oligonucleotides and can be performed *in vitro* or *in vivo*. The dosage of oligonucleotides may be adjusted to optimally reduce expression of a protein translated from a target nucleic acid molecule, *e.g.*, as measured by a readout of RNA stability or by a therapeutic response, without undue experimentation.

For example, expression of the protein encoded by the nucleic acid target can be measured to determine whether or not the dosage regimen needs to be adjusted accordingly. In addition, an increase or decrease in RNA or protein levels in a cell or produced by a cell can be measured using any art recognized technique. By determining whether transcription has been decreased, the effectiveness of the oligonucleotide in inducing the cleavage of a target RNA can be determined.

Any of the above-described oligonucleotide compositions can be used alone or in conjunction with a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" includes appropriate solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, it can be used in the therapeutic compositions. Supplementary active ingredients can also be incorporated into the compositions.

Oligonucleotides may be incorporated into liposomes or liposomes modified with polyethylene glycol or admixed with cationic lipids for parenteral administration. Incorporation of additional substances into the liposome, for example, antibodies reactive against membrane proteins found on specific target cells, can help target the oligonucleotides to specific cell types.

Moreover, the present invention provides for administering the subject oligonucleotides with an osmotic pump providing continuous infusion of such oligonucleotides, for example, as described in Rataiczak *et al.* (1992 *Proc. Natl. Acad. Sci. USA* 89:11823-11827). Such osmotic pumps are commercially available, *e.g.*, from Alzet Inc. (Palo Alto, Calif.). Topical administration and parenteral administration in a cationic lipid carrier are preferred.

With respect to *in vivo* applications, the formulations of the present invention can be administered to a patient in a variety of forms adapted to the chosen route of administration, e.g., parenterally, orally, or intraperitoneally. Parenteral administration, which is preferred, includes administration by the following routes: intravenous; intramuscular; interstitially; 5 intraarterially; subcutaneous; intra ocular; intrasynovial; trans epithelial, including transdermal; pulmonary via inhalation; ophthalmic; sublingual and buccal; topically, including ophthalmic; dermal; ocular; rectal; and nasal inhalation via insufflation.

Pharmaceutical preparations for parenteral administration include aqueous solutions of the active compounds in water-soluble or water-dispersible form. In addition, suspensions 10 of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, or dextran, optionally, the suspension 15 may also contain stabilizers. The oligonucleotides of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the oligonucleotides may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included in the invention.

20 Pharmaceutical preparations for topical administration include transdermal patches, ointments, lotions, creams, gels, drops, sprays, suppositories, liquids and powders. In addition, conventional pharmaceutical carriers, aqueous, powder or oily bases, or thickeners may be used in pharmaceutical preparations for topical administration.

25 Pharmaceutical preparations for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. In addition, thickeners, flavoring agents, diluents, emulsifiers, dispersing aids, or binders may be used in pharmaceutical preparations for oral administration.

For transmucosal or transdermal administration, penetrants appropriate to the barrier 30 to be permeated are used in the formulation. Such penetrants are known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives, and detergents. Transmucosal administration may be through nasal sprays or using suppositories. For oral administration, the oligonucleotides are formulated into conventional oral administration forms such as capsules, tablets, and tonics. For topical administration, the oligonucleotides of the invention are formulated into ointments, salves, gels, or creams as 35 known in the art.

Drug delivery vehicles can be chosen *e.g.*, for *in vitro*, for systemic, or for topical administration. These vehicles can be designed to serve as a slow release reservoir or to deliver their contents directly to the target cell. An advantage of using some direct delivery drug vehicles is that multiple molecules are delivered per uptake. Such vehicles have been  
5 shown to increase the circulation half-life of drugs that would otherwise be rapidly cleared from the blood stream. Some examples of such specialized drug delivery vehicles which fall into this category are liposomes, hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres.

The described oligonucleotides may be administered systemically to a subject.  
10 Systemic absorption refers to the entry of drugs into the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include: intravenous, subcutaneous, intraperitoneal, and intranasal. Each of these administration routes delivers the oligonucleotide to accessible diseased cells. Following subcutaneous administration, the therapeutic agent drains into local lymph nodes and  
15 proceeds through the lymphatic network into the circulation. The rate of entry into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier localizes the oligonucleotide at the lymph node. The oligonucleotide can be modified to diffuse into the cell, or the liposome can directly participate in the delivery of either the unmodified or modified oligonucleotide into the cell.

20 The chosen method of delivery will result in entry into cells. Preferred delivery methods include liposomes (10-400 nm), hydrogels, controlled-release polymers, and other pharmaceutically applicable vehicles, and microinjection or electroporation (for *ex vivo* treatments).

25 The pharmaceutical preparations of the present invention may be prepared and formulated as emulsions. Emulsions are usually heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 µm in diameter.

30 The emulsions of the present invention may contain excipients such as emulsifiers, stabilizers, dyes, fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives, and anti-oxidants may also be present in emulsions as needed. These excipients may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase.

Examples of naturally occurring emulsifiers that may be used in emulsion formulations of the present invention include lanolin, beeswax, phosphatides, lecithin and acacia. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. Examples of finely divided solids that may be used as emulsifiers include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

Examples of preservatives that may be included in the emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Examples of antioxidants that may be included in the emulsion formulations include free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

In one embodiment, the compositions of oligonucleotides are formulated as microemulsions. A microemulsion is a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution. Typically microemulsions are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a 4th component, generally an intermediate chain-length alcohol to form a transparent system.

Surfactants that may be used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (S0750), decaglycerol decaoleate (DA0750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules.

Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C<sub>8</sub>-C<sub>12</sub>) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C<sub>8</sub>-C<sub>10</sub> glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both oil/water and water/oil) have been proposed to enhance the oral bioavailability of drugs.

Microemulsions offer improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides *et al.*, Pharmaceutical Research, 1994, 11:1385; Ho *et al.*, J. Pharm. Sci., 1996, 85:138-143). Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

In an embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to increasing the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also act to enhance the permeability of lipophilic drugs.

Five categories of penetration enhancers that may be used in the present invention include: surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants. Other agents may be utilized to enhance the penetration of the administered oligonucleotides include: glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-15 pyrrol, azones, and terpenes such as limonene, and menthone.

The oligonucleotides, especially in lipid formulations, can also be administered by coating a medical device, for example, a catheter, such as an angioplasty balloon catheter, with a cationic lipid formulation. Coating may be achieved, for example, by dipping the medical device into a lipid formulation or a mixture of a lipid formulation and a suitable 5 solvent, for example, an aqueous-based buffer, an aqueous solvent, ethanol, methylene chloride, chloroform and the like. An amount of the formulation will naturally adhere to the surface of the device which is subsequently administered to a patient, as appropriate. Alternatively, a lyophilized mixture of a lipid formulation may be specifically bound to the 10 surface of the device. Such binding techniques are described, for example, in K. Ishihara *et al.*, Journal of Biomedical Materials Research, Vol. 27, pp. 1309-1314 (1993), the disclosures of which are incorporated herein by reference in their entirety.

The useful dosage to be administered and the particular mode of administration will vary depending upon such factors as the cell type, or for *in vivo* use, the age, weight and the particular animal and region thereof to be treated, the particular oligonucleotide and delivery 15 method used, the therapeutic or diagnostic use contemplated, and the form of the formulation, for example, suspension, emulsion, micelle or liposome, as will be readily apparent to those skilled in the art. Typically, dosage is administered at lower levels and increased until the desired effect is achieved. When lipids are used to deliver the oligonucleotides, the amount of lipid compound that is administered can vary and generally depends upon the amount of 20 oligonucleotide agent being administered. For example, the weight ratio of lipid compound to oligonucleotide agent is preferably from about 1:1 to about 15:1, with a weight ratio of about 5:1 to about 10:1 being more preferred. Generally, the amount of cationic lipid compound which is administered will vary from between about 0.1 milligram (mg) to about 1 gram (g). By way of general guidance, typically between about 0.1 mg and about 10 mg of the 25 particular oligonucleotide agent, and about 1 mg to about 100 mg of the lipid compositions, each per kilogram of patient body weight, is administered, although higher and lower amounts can be used.

The agents of the invention are administered to subjects or contacted with cells in a biologically compatible form suitable for pharmaceutical administration. By "biologically 30 compatible form suitable for administration" is meant that the oligonucleotide is administered in a form in which any toxic effects are outweighed by the therapeutic effects of the oligonucleotide. In one embodiment, oligonucleotides can be administered to subjects. Examples of subjects include mammals, *e.g.*, humans and other primates; cows, pigs, horses, and farming (agricultural) animals; dogs, cats, and other domesticated pets; mice, rats, and 35 transgenic non-human animals.

Administration of an active amount of an oligonucleotide of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, an active amount of an oligonucleotide may vary according to factors such as the type of cell, the oligonucleotide used, and for *in vivo* uses the disease state, 5 age, sex, and weight of the individual, and the ability of the oligonucleotide to elicit a desired response in the individual. Establishment of therapeutic levels of oligonucleotides within the cell is dependent upon the rates of uptake and efflux or degradation. Decreasing the degree of degradation prolongs the intracellular half-life of the oligonucleotide. Thus, chemically-modified oligonucleotides, *e.g.*, with modification of the phosphate backbone, may require 10 different dosing.

The exact dosage of an oligonucleotide and number of doses administered will depend upon the data generated experimentally and in clinical trials. Several factors such as the desired effect, the delivery vehicle, disease indication, and the route of administration, will affect the dosage. Dosages can be readily determined by one of ordinary skill in the art and 15 formulated into the subject pharmaceutical compositions. Preferably, the duration of treatment will extend at least through the course of the disease symptoms.

Dosage regima may be adjusted to provide the optimum therapeutic response. For example, the oligonucleotide may be repeatedly administered, *e.g.*, several doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies 20 of the therapeutic situation. One of ordinary skill in the art will readily be able to determine appropriate doses and schedules of administration of the subject oligonucleotides, whether the oligonucleotides are to be administered to cells or to subjects.

#### Treatment of Diseases or Disorders

25 By inhibiting the expression of a gene, the oligonucleotide compositions of the present invention can be used to treat any disease involving the expression of a protein. Examples of diseases that can be treated by oligonucleotide compositions include: cancer, retinopathies, autoimmune diseases, inflammatory diseases (*i.e.*, ICAM-1 related disorders, Psoriasis, Ulcerative Colitus, Crohn's disease), viral diseases (*i.e.*, HIV, Hepatitis C), and 30 cardiovascular diseases.

In one embodiment, *in vitro* treatment of cells with oligonucleotides can be used for *ex vivo* therapy of cells removed from a subject (e.g., for treatment of leukemia or viral infection) or for treatment of cells which did not originate in the subject, but are to be administered to the subject (e.g., to eliminate transplantation antigen expression on cells to be transplanted into a subject). In addition, *in vitro* treatment of cells can be used in non-therapeutic settings, e.g., to evaluate gene function, to study gene regulation and protein synthesis or to evaluate improvements made to oligonucleotides designed to modulate gene expression or protein synthesis. *In vivo* treatment of cells can be useful in certain clinical settings where it is desirable to inhibit the expression of a protein. There are numerous medical conditions for which antisense therapy is reported to be suitable (see, e.g., U.S. 5,830,653) as well as respiratory syncytial virus infection (WO 95/22,553) influenza virus (WO 94/23,028), and malignancies (WO 94/08,003). Other examples of clinical uses of antisense sequences are reviewed, e.g., in Glaser. 1996. *Genetic Engineering News* 16:1. Exemplary targets for cleavage by oligonucleotides include, e.g., protein kinase Ca, ICAM-1, c-raf kinase, p53, c-myb, and the bcr/abl fusion gene found in chronic myelogenous leukemia.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, J. et al. (Cold Spring Harbor Laboratory Press (1989)); *Short Protocols in Molecular Biology*, 3rd Ed., ed. by Ausubel, F. et al. (Wiley, NY (1995)); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed. (1984)); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. (1984)); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London (1987)); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds. (1986)); and Miller, J. *Experiments in Molecular Genetics* (Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1972)).

The invention is further illustrated by the following examples, which should not be construed as further limiting.

## EXAMPLES

Example 1. Oligonucleotide Compositions Comprising Chimeric Antisense Sequences.

A gapped antisense oligonucleotide comprising 2'-O-methyl RNA arms and an  
5 unmodified DNA gap was synthesized. A complementary oligonucleotide was also synthesized using unmodified RNA. A double-stranded duplex was formed and the composition was found to inhibit expression of the target gene.

Example 2. Length of Double-Stranded Oligonucleotides and the Presence or Absence of Overhangs Has No Effect on Function.

Twenty one and 27-mers were designed to target each of two sites on the p53 molecule (89-90 site, and 93-94 site). The double-stranded molecules were designed with or without 3'-deoxy TT overhangs. The test oligonucleotides were 21-mers with 2 nucleotide 3'  
10 deoxy TT overhangs and without overhangs (blunt ends); and 27-mers with 2 nucleotide 3' deoxy TT overhangs and without overhangs (blunt ends). Two positive controls were included in the experiment (p53) and two negative controls were also included (FITC)

A549 cells were transfected with 100 nM of the double-stranded molecules plus 2 ug/mL Lipofectamine 2000. A549 cells were examined 24 hours post-transfection. FITC-labeled molecules were taken up well by cells. Both 21-mers (with or without overhangs)  
20 and 27-mers (with or without overhangs) were non-toxic to cells. Figure 1 shows the result of an experiment comparing the ability of different oligonucleotide constructs to inhibit p53 and shows that length or the presence or absence of a 3' deoxy TT overhang did not affect the activity of the oligonucleotide. The results in Figure 1 show the amount of p53 mRNA normalized to the amount of an irrelevant message, GAPDH. The level of mRNA was  
25 determined using RT-PCR analysis. The observed percent inhibition of p53 expression is shown below:

SITE	21-MER		27-MER	
	overhang	no overhang	overhang	no overhang
93-94	58%	65%	62 %	62%
89-90	81%	75%	67%	70%

Similar results were observed for  $\beta$ -3-integrin; both 21-mer and 27-mer double-stranded molecules were found to inhibit integrin mRNA. Two double-stranded RNA complexes designed to target the same site of the  $\beta$ -3-integrin gene were transfected in HMVEC cells. Both complexes contained a two nucleotide (TT) overhang: one complex was 5 a 21-mer (with 19 nucleotides complementary to the target gene) and the other was a 27-mer (with 25 nucleotides complementary to the target gene). RT-PCR analysis showed that the two complexes inhibited the target gene to the same extent. HMVEC cells were transfected using 100 nM oligomer complexed with 2ug/mL of Lipofectamine 2000 in media containing serum for 24 hours. Twenty-four hours after transfection, the cells were lysed and the RNA 10 was isolated for analysis by RT-PCR. No significant toxicity was observed. The results in Figure 1B show the amount of  $\beta$ -3-integrin mRNA normalized to the amount of GAPDH, as determined by RT-PCR analysis.

15 Example 3. Activation of the Double-Stranded RNA, Interferon-Inducible Protein Kinase, PKR.

PKR is activated by double-stranded RNA molecules. Active PKR leads to the inhibition of protein synthesis, activation of transcription, and a variety of other cellular effects, including signal transduction, cell differentiation, cell growth inhibition, apoptosis, and antiviral effects. The effect of p53-targeted double-stranded RNA molecules on PKR 20 expression was tested. The level of mRNA was determined using RT-PCR analysis. As shown in Figure 2, no correlation was observed between the length of the double-stranded oligonucleotide and the level of PKR induction. Accordingly, long oligonucleotides can be used without activating PKR, a marker for interferon induction.

As illustrated in Figure 2B, analysis of relative amounts of PKR mRNA after the 21- 25 and 27-mer transfection in HMVEC cells showed approximately a 2 fold increase in PKR mRNA of the siRNA control sequences over no treatment, and approximately a 2 fold increase of PKR mRNA of the 27-mer compared to the 21-mer targeted double-stranded RNA complexes.

Example 4. The Effect of 5' vs. 3' Modification on the Activity of Double-Stranded Oligonucleotides.

Oligonucleotide duplexes were modified at either the 3' or 5' end with FITC groups. The modifications were made on either the antisense strand or the sense strand. 5' or 3' modification of the sense strand had no effect on the percent inhibition of p53 mRNA. 3' modification of the antisense strand had little affect on activity, while 5' modification of the antisense strand reduced activity significantly. 3' modification of both strands also had little affect on activity, while 3' and 5' modification of both strands reduced activity. See Figure 3.

The effect of the size of the group used to modify the 5' end was tested. The results of this experiment are shown in Figure 4. The inclusion of a 5' phosphate group had little affect on activity, whereas the modification of the antisense strand or both strands had a greater effect. The inclusion of a propyl group had more of an effect, with a 5' propyl group on the antisense strand showing a large reduction in activity; there was also an effect when this group was added to both strands. Similarly, the inclusion of a FITC group at the 5' end of the antisense molecule (or to both molecules) also significantly reduced the activity of the RNA duplex.

Example 5. Comparison of the Efficacy of 2'-O-Me Modified and Unmodified Double-Stranded RNA Oligonucleotides.

A549 cells were transfected with modified or unmodified RNA duplexes complexed at 100 nM with 2 ug/mL Lipofectamine 2000 (Invitrogen) and were transfected for 24 hours. The A549 cells were plated at 20,000/well in 48 well plates. After 24 hours, FITC-labeled double-stranded oligonucleotides were visible in A549 cells; the inclusion of a 2'-O-Me group did not affect uptake. The Table below shows the results of this experiment.

25

2'-O-Me Oligonucleotide Duplexes				
	Anti-sense/Sense	Anti-sense/Sense	Anti-sense/Sense	Anti-sense/Sense
	2'-O-Me/2'-O-Me	2'-O-Me/RNA	RNA/2'-O-Me	RNA/RNA
targeted	18639/18640	18639/16194	16193/18640	18876
non-targeted	19039/19040	19039/19044	19043/19040	18850 & 16197/16198
	FITC-2'-O-Me/ FITC 2'-O-Me	FITC-2'-O-Me/ FITC-RNA	FITC-2'-O-Me/ RNA	2'-O-Me/ FITC-RNA
non-targeted	19209	19037/19042	19037/19044	19039/19042

The effect of 2'-O-Me modifications to one or both strands of a double-stranded RNA molecule is shown in Figure 5.

#### 5 Example 6. Toxicity of p53-Targeted siRNAs in A549 Cells.

27-mer siRNAs targeting p53 were not toxic to cells when compared to standard 21-mer siRNAs having 3' deoxy TT overhangs. In this experiment, both siRNA constructs inhibited p53 to a similar extent (83% inhibition for 27-mer vs. 90% inhibition for 21-mer).  
10 siRNAs were designed to target p53 and were constructed as blunt-end 27-mers or as 21-mers with 3' deoxy TT overhangs. A549 cells were plated at 20,000 cells per well in 48-well plates on the day prior to transfection. On the day of transfection, cells were approximately 60-70% confluent. Cells were transfected with 100 nM siRNAs complexed with 2 ug / mL Lipofectamine 2000 for 24 hours. Following transfection, cells were stained with Dead Red stain to visualize the extent of cell death. The siRNA sequences used were as follows:

15

### 21-mer with overhangs targeted (5'-3'):

ACCUCAAAGCUGUUCCGUU  
GACGGAACAGCUUUGAGGU (SEQ ID NO: ##)

### Blunt-end 27-mer targeted (5'-3'):

ACGCACACCUAAAGCUGUUCGUCCC (SEQ ID NO: ##)  
GGGACGGAACAGCTTGAGGTGTGCGT (SEQ ID NO: ##)

Example 7. Toxicity of Blunt-End 27-mer siRNAs Targeting p53 in A549 Cells.

The toxicity of targeted blunt-end 27-mer siRNAs targeting p53 was observed to be not significantly different than a control nucleic acid or no treatment. siRNAs were designed to target p53 and were constructed as blunt-end 27-mers. The corresponding control consisted of chemistry-matched, scrambled sequences with a similar base-pair composition. A549 cells were plated at 20,000 cells per well in 48-well plates on the day prior to transfection. On the day of transfection, cells were approximately 60-70% confluent. Cells were transfected with 100 nM siRNAs complexed with 2 ug / mL Lipofectamine 2000 for 24 hours. Following transfection, the cells were stained with Dead Red stain to visualize the extent of cell death. The siRNA sequences used were as follows:

Blunt-end 27-mer targeted (5'-3' on top):

ACGCACACCUCAAAGCUGUUCCGUCCC (SEQ ID NO: ##)  
GGGACGGAACAGCTTGAGGTGTGCGT (SEQ ID NO: ##)

Corresponding control (5'-3' on top):

CCCTGCCTTGTGAAACTCCACACGCA (SEQ ID NO: ##)  
TGCCTGTGGAGTTCGACAAGGCAGGG (SEQ ID NO: ##)

Example 8. Toxicity of Blunt End 32-mer siRNAs Targeting p53 in A549 Cells.

Similarly, blunt-end 32-mer siRNAs targeting p53 were not observed to be toxic to cells in comparison with a control nucleic acid and no treatment, as determined by Dead Red staining. siRNAs were designed to target p53 and were constructed as blunt-end 32-mers. The corresponding control consisted of chemistry-matched, scrambled sequences with a similar base-pair composition. A549 cells were plated at 20,000 cells per well in 48-well plates on the day prior to transfection. On the day of transfection, cells were approximately 60-70% confluent. Cells were transfected with 100 nM siRNAs complexed with 2 ug / mL Lipofectamine 2000 for 24 hours. Following transfection, cells were stained with Dead Red stain to visualize the extent of cell death. The siRNA sequences used were as follows:

Targeted blunt-end 32-mer (5'-3' on top):

CCCTCACGCACACCUCAAAGCUGUUCCGUCCC (SEQ ID NO: ##)  
GGGACGGAACAGCTTGAGGTGTGCGTGAGGG (SEQ ID NO: ##)

Corresponding control (5'-3' on top):

CCCTGCCTTGTGAAACTCCACACGCACTCCC (SEQ ID NO: ##)  
GGGAGTGCCTGTGGAGTTCGACAAGGCAGGG (SEQ ID NO: ##)

Example 9. Inhibition of p53 by 32- and 37-mer Blunt-End siRNAs.

Figure 6 depicts the results of inhibition of p53 by 32- and 37-mer blunt-end siRNAs in comparison with various control experiments. siRNAs were designed to target each of two sites (93-93 site) and (89-90 site) along the coding region of p53. siRNAs were constructed as blunt-end 32-mers or blunt-end 37-mers. Positive control siRNAs were 21-mers with 3' deoxy TT overhangs. Corresponding controls consisted of chemistry-matched, scrambled sequences with a similar base-pair composition. A549 cells were plated at 20,000 cells per well in 48-well plates on the day prior to transfection. On the day of transfection, cells were approximately 60-70% confluent. Cells were transfected with 100 nM siRNAs complexed with 2 ug / mL Lipofectamine 2000 for 24 hours. Following transfection, cells were lysed and poly(A) mRNA was harvested for RT-PCR. Inhibition of p53 expression was determined by quantitative real-time RT-PCR (TaqMan) analysis. Expression of p53 was standardized by quantifying GAPDH for each sample. The data in Figure 6 represent three separate transfections analyzed in duplicate and normalized to the internal control (GAPDH).

15 The siRNA sequences used were as follows (depicted with the 5'-3' strand on top):

## Targeted 32-mer (89-90 site):

CCCTCACGCACACCUCAAAGCUGUUCGUCCC	(SEQ ID NO: ##)
GGGACGGAACAGCTTGAGGTGTGCGTGAGGG	(SEQ ID NO: ##)

## 32-mer control (89-90 site):

CCCTGCCTTGTGAAACTCCACACGCACACTCCC	(SEQ ID NO: ##)
GGGAGTGCCTGTGGAGTTCGACAAGGGCAGGG	(SEQ ID NO: ##)

## 32-mer targeted (93-94 site):

CCCUUCUGUCUUGAACAUAGAGTTTTATGGC	(SEQ ID NO: ##)
GCCATAAAAAACTCATGTTCAAGACAGAAGGG	(SEQ ID NO: ##)

## 32-mer control (93-94 site):

CGGTATTTTGAGTACAAGTTCTGTCTTCCC	(SEQ ID NO: ##)
GGGAAGACAGAACATTGTACTCAAAAAATACCG	(SEQ ID NO: ##)

## 37-mer targeted (93-94 site):

CCCTTCTGTCTTGAACATGAGTTTTATGGCGGGAG	(SEQ ID NO: ##)
CTCCCGCCATAAAAACATGTTCAAGACAGAAGGG	(SEQ ID NO: ##)

## 37-mer control (93-94 site):

GAGGGCGGTATTTTGAGTACAAGTTCTGTCTTCCC (SEQ ID NO: ##)  
 GGGAAAGACAGAACTTGTACTCAAAAAATACCGCCCTC (SEQ ID NO: ##)

## 21-mer targeted (89-90 site):

ACCUCAAAGCUGUUCCGUCTT (SEQ ID NO: ##)  
 GACGGAACAGCUUJUGAGGUTT (SEQ ID NO: ##)

## 21-mer targeted (93-94 site):

CCUUCUGUCUUGAACAU GTT (SEQ ID NO: ##)  
 CAUGUCAAGACAGAAGGGTT (SEQ ID NO: ##)

Example 10. Enhanced Cellular Stability of Double-Stranded 2'-O-Methyl RNA.

In this example, the single-stranded control oligomer was transfected at 800 nM.

- 5 Accumulation was observed in the nucleus at 6 hours post transfection, however by 25 hours the fluorescence of the single-stranded oligomer had largely dissipated, indicating the oligomer was no longer intact (Fisher, T., T. Terhorst, et al. (1993). "Intracellular disposition and metabolism of fluorescently-labeled unmodified and modified oligonucleotides microinjected into mammalian cells." NAR 21: 3857-3865). The relative fluorescence of  
 10 fluoresceinyl-labeled oligomers transfected into A549 cells was observed to fit the following pattern:

	single-stranded (800 nM)	double-stranded (100 nm)
6 h	++++	+++++
25 h	+	+++++

- 15 The double-stranded oligomer duplex, wherein the second strand was 2'-O-methyl modified RNA, was transfected at 100nM, and was also clearly visible at 6 hours post transfection. However, in contrast to the single-stranded oligomer, the double-stranded was still largely intact in the nucleus at 24 hours, even though the concentration transfected was 8-fold less, thereby demonstrating that the 2'-O-methyl second strand stabilized the oligomer in the cell.

The oligomers were all 2'-O-CH<sub>3</sub> with a phosphodiester backbone containing 6-carboxyfluorescein (6-FAM) tethered to the 5' hydroxyl. The single-stranded control oligomer was transfected at 800nM complexed with 4 ug/mL of Lipofectamine 2000, and the double-stranded complex was transfected at 100 nM complexed with 1 ug/mL of  
5 Lipofectamine 2000.

Fluorescent signal was seen accumulating in the nucleus at 6 hours post transfection, however by 24 hours the single-stranded oligomer has significantly dissipated, indicating the oligomer is no longer intact. The double-stranded duplexes (wherein the second strand is 2'-O-methyl modified RNA with a 5' 6-FAM) was transfected at 100nM, and was also clearly  
10 visible at 6 hours post transfection. In contrast to the single-stranded oligomer, the double-stranded was still largely intact in the nucleus at 24 hours, even though the concentration transfected was 8-fold less. This experiment demonstrates that the 2'-O-methyl second strand stabilizes the duplex in the cell.

15 Example 11. Enhanced Stability in Cells and Accumulation in Cytoplasm of RNA Hybridized to 2'-O-Methyl RNA.

The fluorescence signal, corresponding to uptake of FITC-labeled RNA and 2'-O-methyl modified RNA duplexes, was measured at 6 and 24 hours. RNA complexes were transfected in A549 cells with 100 nM oligomer complexed with 2ug/mL Lipofecatmine  
20 2000 as described below. Cells were continuously transfected for 24 hours and fluorescent uptake was assessed at 6 and 24 hours. Oligomers were 2'-O-methyl modified RNA with 5' 6-FAM (FITC-2'-OMe), 19-mer RNA with two deoxynucleotides on the 3' end with 5' 6-FAM (FITC-RNA) or 19-mer RNA with two deoxynucleotides on the 3' end (RNA)  
25 complexed. At 6 hours, the FITC-2'-O-methyl duplexes show localization in the nucleus and the FITC-2'-O-methyl/RNA and 2'-O-methyl/FITC-RNA complexes show a more diffuse pattern of uptake (these RNA/2'-O-methyl complexes are a substrate for the RISC complex and are therefore retained in the cytoplasm where the RISC complex has been reported to be active). At 24 hours, the FITC-2'-O-methyl/RNA and 2'-O-methyl/FITC-RNA complexes were still visible in the cell, whereas typically not even the single-stranded FITC-2'-O- was  
30 visible, even when transfected at significantly higher concentrations, demonstrating that the 2'-O-methyl RNA protects the RNA strand from degradation in the cell.

RNA oligomers having a phosphodiester backbone with 2'-O-methyl nucleotides were synthesized using standard phosphoramidite chemistry. Oligomers were purified by denaturing polyacrylamide gel electrophoresis (PAGE). Purity of oligomers was confirmed by (PAGE) and mass spectrometry. All oligomers were greater than 90% full length, and 5 mass data obtained was consistent with expected values. Target-specific siRNA duplexes consisted of 21-nt sense and 21-nt antisense strands with symmetric 2-nt 3' deoxy TT overhangs. 21-nt RNAs were chemically synthesized using phosphoramidite chemistry. For duplex preparation, sense- and antisense oligomers (each at 50 µM) were combined in equal 10 volumes in annealing buffer (30 mM HEPES pH 7.0, 100 mM potassium acetate, and 2 mM magnesium acetate), heat-denatured at 90 °C for 1 min and annealed at 37 °C for one hour. Duplexes were stored at 80 °C until used.

A549 cells (ATCC #CCL-185) were cultured at 37 °C in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies #11960-044) supplemented with 2 mM L-glutamine, 100 units / mL penicillin, 100 µg / mL streptomycin, and 10% fetal bovine serum (FBS). 15 HeLa cells (ATCC #CCL-2) were cultured at 37 °C in Minimal Essential Medium (MEM, Life Technologies #10370-021) supplemented with 2 mM L-glutamine, 1.5 g / L sodium bicarbonate, 1.0 mM sodium pyruvate, 100 units / mL penicillin, 100 µg / mL streptomycin, and 10% FBS. Cells were passaged regularly to maintain exponential growth. On the day prior to transfection, cells were trypsinized, counted, and seeded in 48-well plates at a density 20 of 20 x 10<sup>3</sup> cells per well in 250 µL fresh media. On the day of transfection cells were typically 60-65% confluent. Transfection of siRNA duplexes and oligomers was carried out using Lipofectamine 2000 (Life Technologies). Briefly, a 10X stock of Lipofectamine 2000 was prepared in Opti-Mem (Life Technologies) and incubated at room temperature for 15 minutes. An equal volume of a 10X stock of siRNA duplex or oligomers in Opti-Mem was 25 added and complexation carried out for 15 minutes at room temperature. Complexes were then diluted 5-fold in full growth media. Culture media was removed from each well prior to the addition of 250 µL complexes per well. Cells were incubated at 37 °C/5% CO<sub>2</sub> for 6 or 24 hours prior to assessing the uptake .

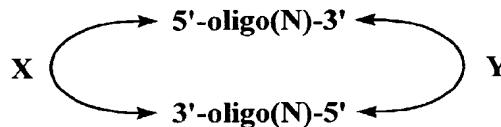
30 **Equivalents**

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. The entire contents of all patents, published patent applications and other references cited 35 herein are hereby expressly incorporated herein in their entireties by reference.

## CLAIMS

1. A double-stranded oligonucleotide composition having the structure:

5



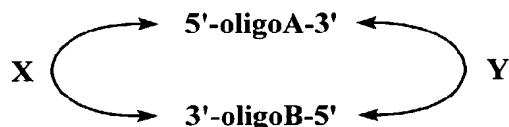
oligo(N) =  
 $(N)_{12-40}$

wherein

(1) N is a nucleomonomer in complementary oligonucleotide strands of equal length  
10 and where the sequence of Ns corresponds to a target gene sequence and

(2) X and Y are each independently selected from a group consisting of nothing; from about 1 to about 20 nucleotides of 5' overhang; from about 1 to about 20 nucleotides of 3' overhang; and a loop structure consisting from about 4 to about 20  
15 nucleomonomers, where the nucleomonomers are selected from the group consisting of G and A.

2. A double-stranded oligonucleotide composition having the structure:



20

wherein

(1) oligoA is an oligonucleotide of a number of nucleomonomers;

(2) oligoB is an oligonucleotide that has the same number of nucleomonomers as oligoA and that is complementary to oligoA;

25

(3) either oligoA or oligoB corresponds to a target gene sequence;

(4) X is selected from a group consisting of (a) nothing; (b) an oligonucleotide of about 1 to about 20 nucleotides covalently bonded to the 5' end of oligoA and constituting a 5' overhang; (c) an oligonucleotide of about 1 to about 20 nucleotides covalently bonded to the 3' end of oligoB and constituting a 3' overhang; (d) and an oligonucleotide of about 4 to about 20 nucleomonomers covalently bonded to the 3' end of oligoB and the 5' end of oligoA and constituting a loop structure, where the nucleomonomers are selected from the group consisting of G and A and

(5) Y is selected from a group consisting of (a) nothing; (b) an oligonucleotide of about 1 to about 20 nucleotides covalently bonded to the 5' end of oligoB and constituting a 5' overhang; (c) an oligonucleotide of about 1 to about 20 nucleotides covalently bonded to the 3' end of oligoA and constituting a 3' overhang; (d) and an oligonucleotide of about 4 to about 20 nucleomonomers covalently bonded to the 3' end of oligoA and the 5' end of oligoB and constituting a loop structure, where the nucleomonomers are selected from the group consisting of G and A.

15

3. The composition of claim 1, wherein the number of nucleomonomers in each strand of the duplex is between about 12 and about 40.

20 4. The composition of claim 2, wherein the number of nucleomonomers in each strand oligoA and oligoB is between about 12 and about 40.

5. The composition of claim 1, wherein the number of nucleomonomers in each strand of the duplex is about 27.

25 6. The composition of claim 2, wherein the number of nucleomonomers in each strand of oligoA and oligoB is about 27.

7. The composition of claim 1, wherein X is a sequence of about 4 to about 20 nucleomonomers which form a loop, wherein the nucleomonomers are selected from the group consisting of G and A.

30 8. The composition of claim 2, wherein X or Y is a sequence of about 4 to about 20 nucleomonomers that forms a loop, wherein the nucleomonomers are selected from the group consisting of G and A.

9. The composition of claim 8, wherein two of the adjacent Ns are unlinked.
10. The composition of claim 8, wherein the nucleotide sequence of the loop is GAAA.

5

11. A double-stranded oligonucleotide composition having the structure:

$$5'-(Z)_{2-8}-(N)_{15-40}-(M)_{2-8}-3'$$
$$3'-(Z)_{2-8}-(N)_{15-40}-(M)_{2-8}-5'$$

10

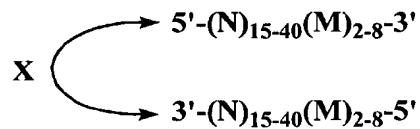
wherein

- (1) each of N, Z, and M is independently a nucleomonomer;
- (2) both of the sequences of Ns are complementary oligonucleotide strands of equal length having between about 15 and about 40 nucleomonomers;
- (3) at least one of the sequences of Ns, optionally with some or all of the flanking Ms or Zs, corresponds to a target gene sequence;
- (4) both of the sequences of Zs are complementary oligonucleotide strands of between about 2 and about 8 nucleomonomers in length; and
- (5) both of the sequences of Ms are complementary oligonucleotide strands of between about 2 and about 8 nucleomonomers in length.

12. The composition of claim 11, wherein each Z and M nucleomonomer is selected from the group consisting of C and G.

- 25 13. The composition of claim 12 wherein, the sequence of Zs or Ms is CC, GG, CG, GC, CCC, GGG, CGG, GCC, GCG, CGC, CGGG, GCCC, CCCC, GGGG, GCGC, CGCG, GGGC, CCCC, CGGG, GCCC, GGCC, or CCGG.

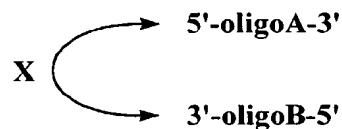
14. A double-stranded oligonucleotide composition having the structure:



wherein

- 5 (1) N is a nucleomonomer in complementary oligonucleotide strands of equal length and where the sequence of Ns corresponds to a target gene sequence and
- (2) X is selected from the group consisting of nothing; 1-20 nucleotides of 5' overhang; 1-20 nucleotides of 3' overhang; a loop structure consisting of from about 4 to about 20 nucleomonomers, where the nucleomonomers are selected from the group consisting of G and A, and
- 10 (3) where M is a nucleomonomer in complementary oligonucleotide strands of between about 2 and about 8 nucleomonomers in length which optionally correspond to the target sequence.

15 15. A double-stranded oligonucleotide composition having the structure:



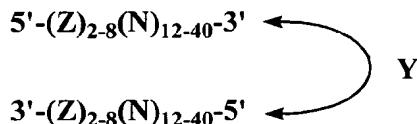
wherein

- 20 (1) oligoA is 5'-(N)<sub>15-40</sub>-(M)<sub>2-8</sub>-3' and oligoB is 5'-(N)<sub>15-40</sub>-(M)<sub>2-8</sub>-3', wherein each of N and M is independently a nucleomonomer;
- (2) both of the sequences of Ns are complementary oligonucleotide strands of equal length having between about 15 and 40 nucleomonomers;
- (3) at least one of the sequences of Ns, optionally with some or all of the flanking Ms, corresponds to a target gene sequence;
- 25

- (4) X is selected from a group consisting of (a) nothing; (b) an oligonucleotide of about 1 to about 20 nucleotides covalently bonded to the 5' end of oligoA and constituting a 5' overhang; (c) an oligonucleotide of about 1 to about 20 nucleotides covalently bonded to the 3' end of oligoB and constituting a 3' overhang; (d) and an oligonucleotide of about 4 to about 20 nucleomonomers covalently bonded to the 3' end of oligoB and the 5' end of oligoA and constituting a loop structure, where the nucleomonomers are selected from the group consisting of G and A; and
- 5 (5) both of the sequences of Ms are complementary oligonucleotide strands of between about 2 and about 8 nucleomonomers in length .

10

16. The composition of claim 15, wherein M nucleomonomer is selected from the group consisting of contain C and G.
- 15 17. The composition of claim 16, wherein the sequence of M is CC, GG, CG, GC, CCC, GGG, CGG, GCC, GCG, CGC, CGGG, GCCC, CCCC, GGGG, GCGC, CGCG, GGGC, CCCG, CGGG, GCCC, GGCC, or CCGG.
18. A double-stranded oligonucleotide composition having the structure:

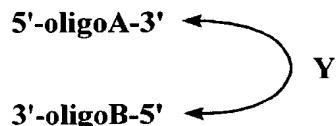


20

wherein

- (1) N is a nucleomonomer in complementary oligonucleotide strands of equal length and which correspond to a target gene sequence and
- 25 (2) Y is selected from the group consisting of nothing; 1-20 nucleotides of 5' overhang; 1-20 nucleotides of 3' overhang; a loop consisting of a sequence of from about 4 to about 20 nucleomonomers, where the nucleomonomers are all either Gs or A's and
- (3) where Z is a are nucleomonomer in complementary oligonucleotide strands of between about 2 and about 8 nucleomonomers in length and which comprise a sequence which can optionally correspond to the target sequence.
- 30

19. A double-stranded oligonucleotide composition having the structure:



5

wherein

(1) oligoA is 5'-(Z)<sub>2-8</sub>-(N)<sub>12-40</sub>-3' and oligoB is 5'-(Z)<sub>2-8</sub>-(N)<sub>12-40</sub>-3', wherein each of N and Z is independently a nucleomonomer;

(2) both of the sequences of Ns are complementary oligonucleotide strands of equal length having between about 12 and 40 nucleomonomers;

10 (3) at least one of the sequences of Ns, optionally with some or all of the flanking Zs, corresponds to a target gene sequence;

(4) Y is selected from a group consisting of (a) nothing; (b) an oligonucleotide of about 1 to about 20 nucleotides covalently bonded to the 5' end of oligoB and constituting a 5' overhang; (c) an oligonucleotide of about 1 to about 20 nucleotides covalently bonded to the 3' end of oligoA and constituting a 3' overhang; (d) and an oligonucleotide of about 4 to about 20 nucleomonomers covalently bonded to the 3' end of oligoA and the 5' end of oligoB and constituting a loop structure, where the nucleomonomers are selected from the group consisting of G and A; and

15 (5) both of the sequences of Zs are complementary oligonucleotide strands of between about 2 and about 8 nucleomonomers in length.

20. The composition of claim 19, wherein the Z nucleomonomers are selected from the group consisting of C and G.

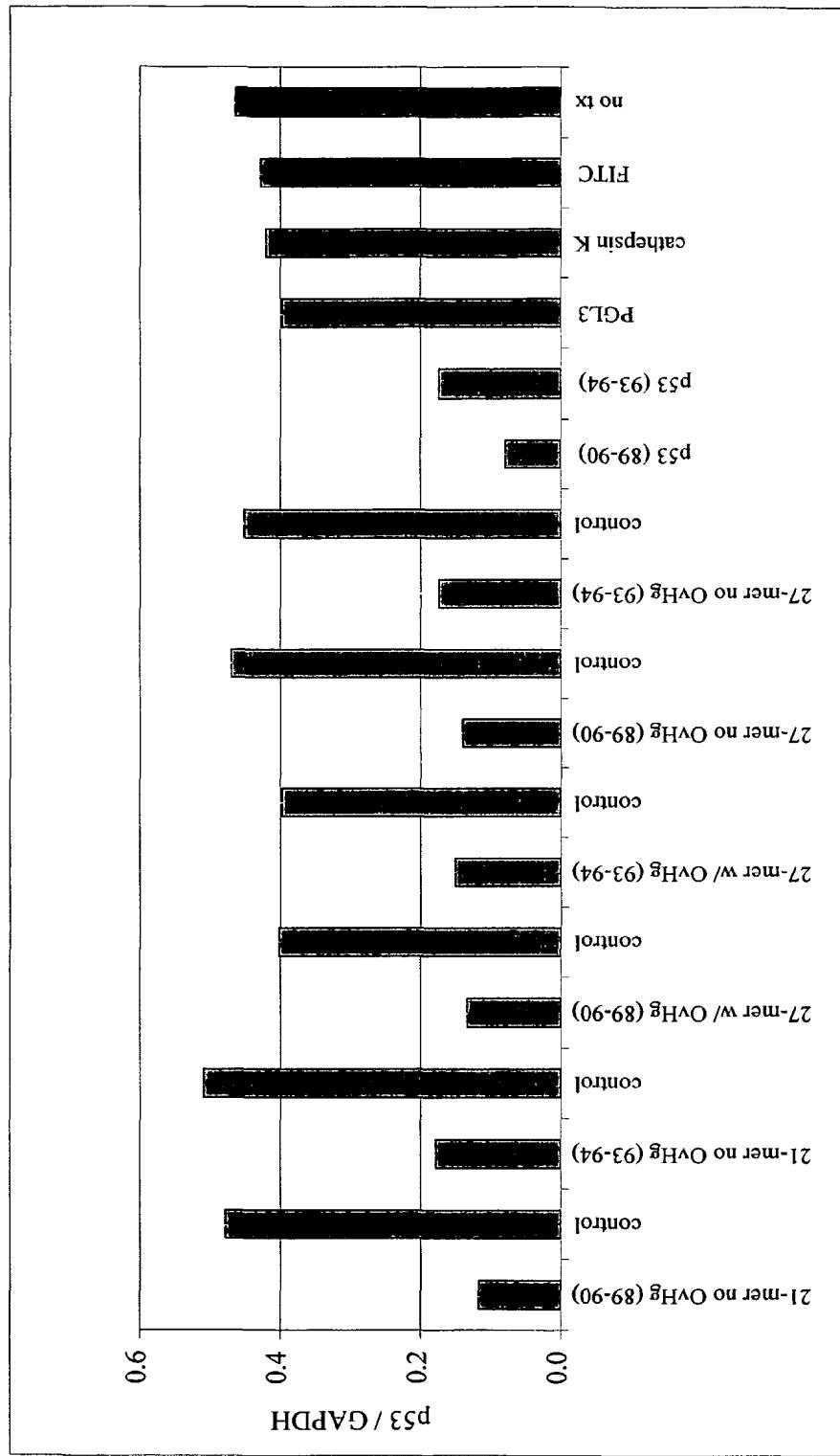
25

21. The composition of claim 20, wherein the sequence of Z is CC, GG, CG, GC, CCC, GGG, CGG, GCC, GCG, CGC, CGGG, GCCC, CCCC, GGGG, GCGC, CGCG, GGGC, CCCG, CGGG, GCCC, GGCC, or CCGG.

22. A method of regulating gene expression in a cell, comprising contacting a cell with the double-stranded duplex oligonucleotide composition of claim 1, to thereby regulate gene expression in a cell.
- 5      23. A method of increasing the nuclease resistance of an antisense sequence, comprising forming a double-stranded oligonucleotide composition of claim 1, such that a double-stranded duplex is formed, wherein the nuclease resistance of the antisense sequence is increased compared to a control composition.

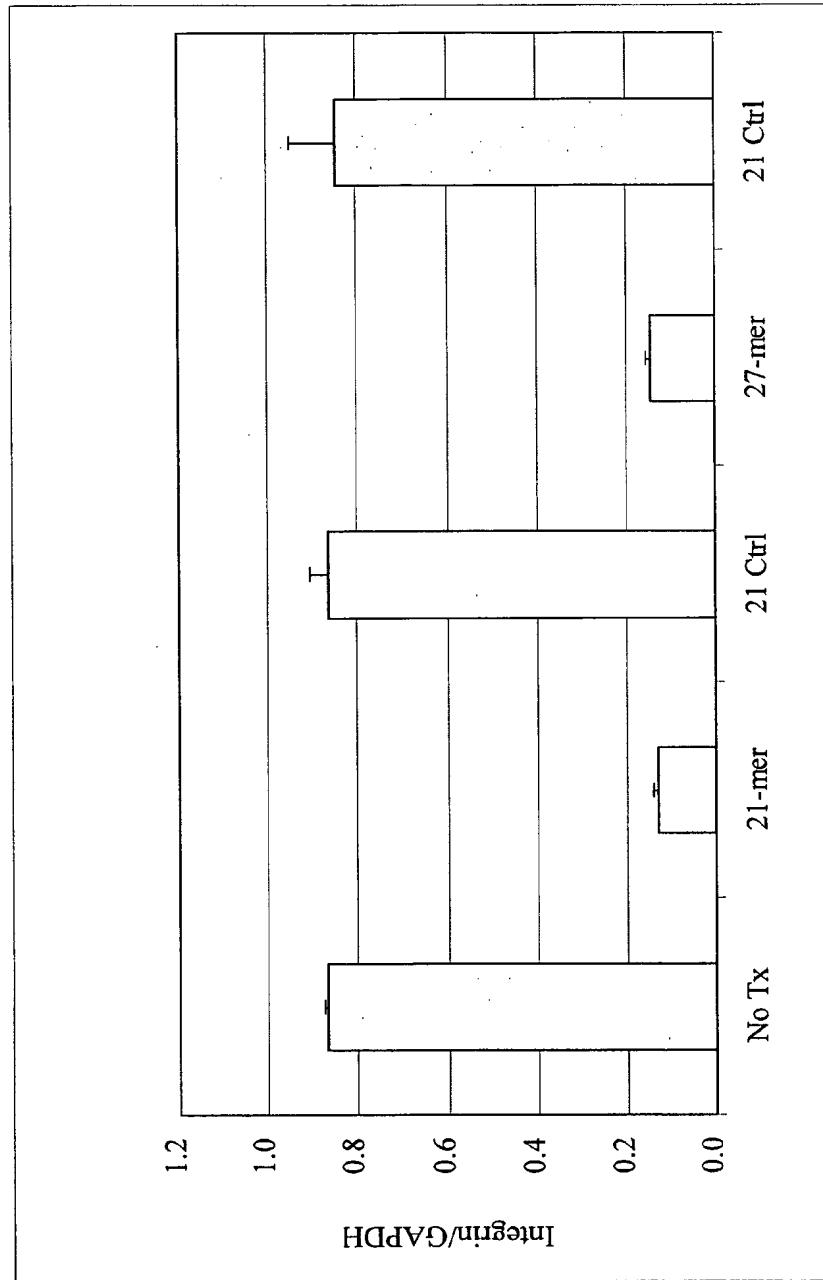
**FIGURE 1.**

*Effect of structural changes on efficacy of siRNAs targeting p53: 21-mers vs. 27-mers with and without 3'-deoxy TT overhangs.*

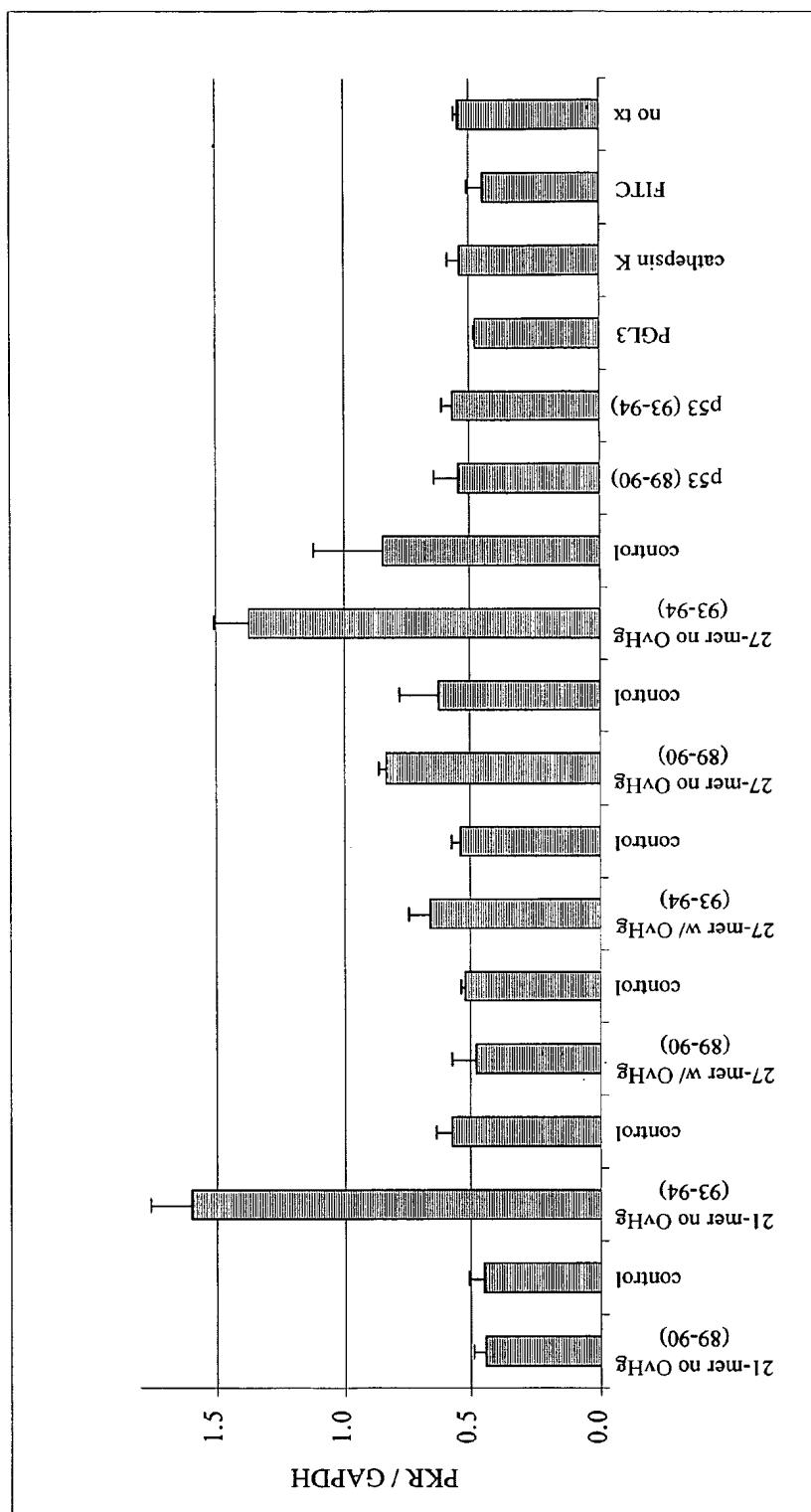


**FIGURE 1B.**

*Effect of structural changes on the efficacy of siRNAs targeting  $\beta$ -3-integrin: 21-mer v. 27-mer.*

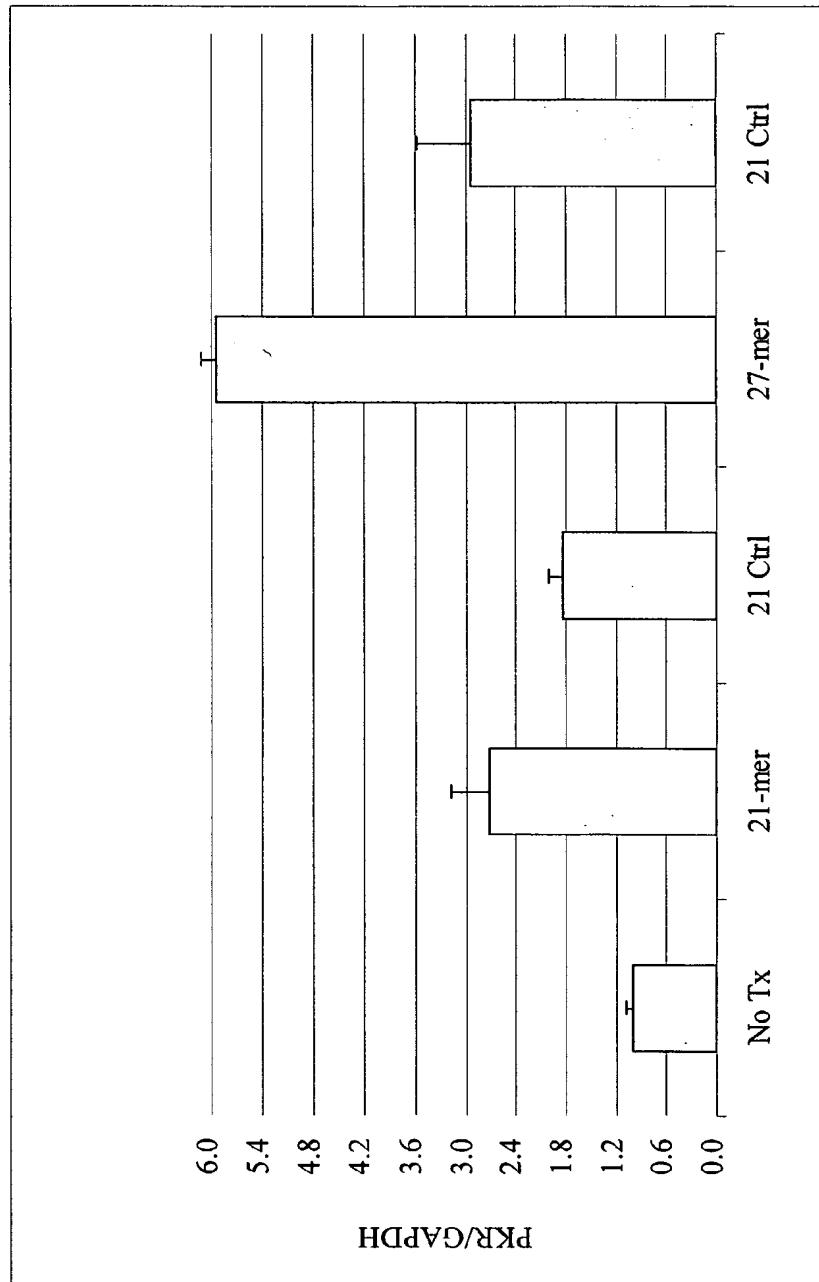


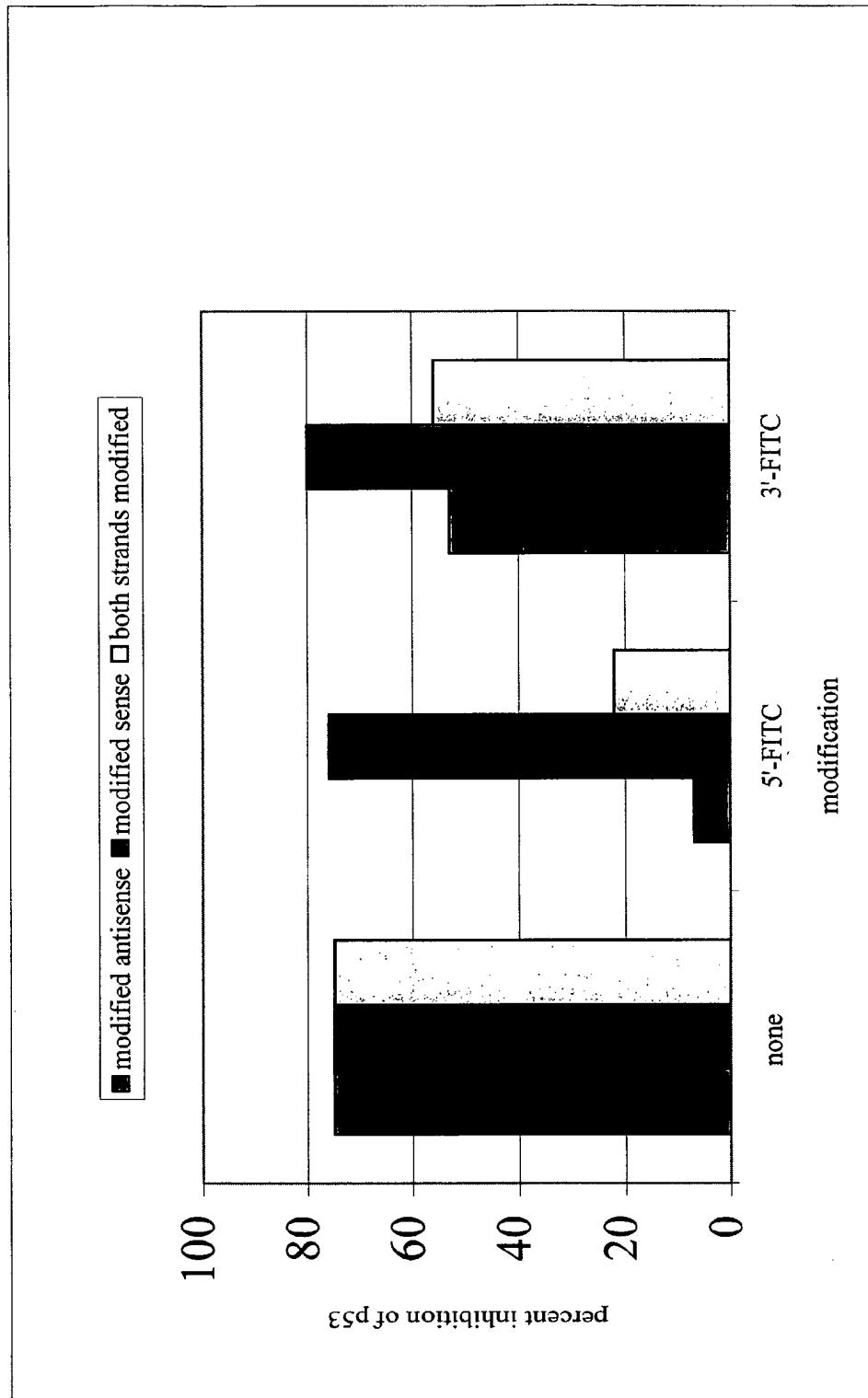
**FIGURE 2.**  
*Effect of p53-targeted and non-targeted siRNAs on PKR expression in A549 cells.*



**FIGURE 2B.**

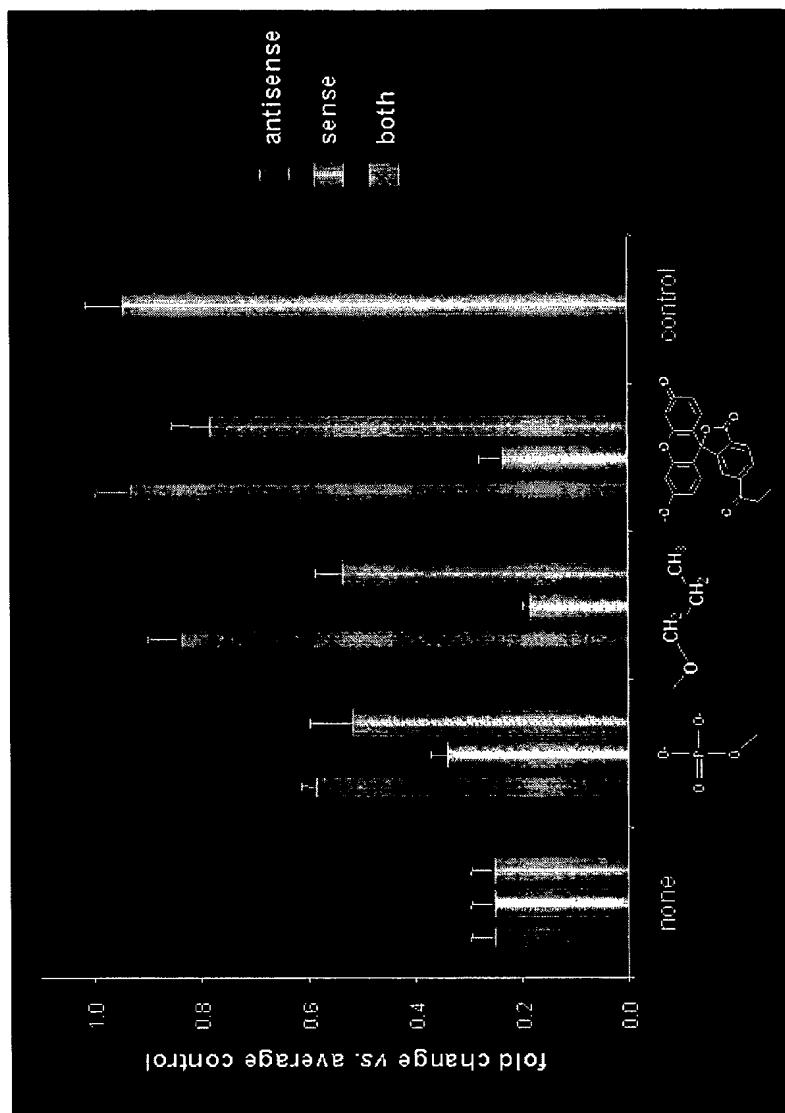
*Effect of  $\beta$ -3-integrin targeted 21-mer and 27-mers on PKR expression in HMVEC cells.*



**FIGURE 3.***Effect of 5' vs. 3' modification on activity.*

**FIGURE 4.**

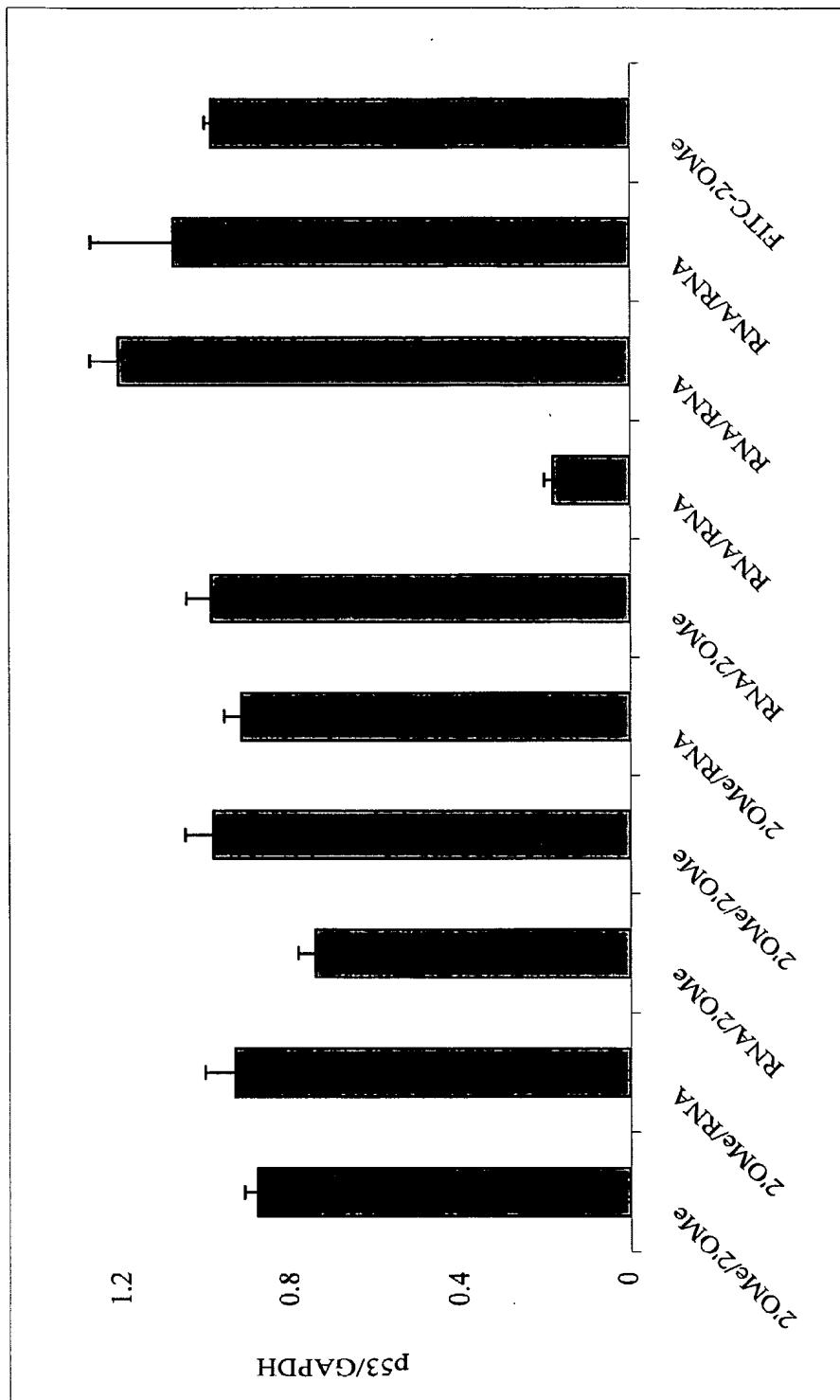
*Effect of the size of the modifying group on activity of the double-stranded RNA duplex.*



**FIGURE 5.**

*Effect of 2'OMe modified siRNAs on p53 expression.*

*(Left to right: first three targeted, next three matched controls, then one targeted, and three additional controls.)*



**FIGURE 6.**  
*Inhibition of p53 by 32- and 37-mer blunt-end siRNAs.*

